Modulation of P-glycoprotein by Zosuquidar Trihydrochloride

Thesis Submitted for the Degree of Doctor of Philosophy

By

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Abstract

P-glycoprotein (P-gp), a major contributor in multidrug resistance (MDR), is a cell surface drug efflux pump restricting the intracellular accumulation of many agents used in cancer chemotherapy leading to treatment failure. Over-expression of P-gp is a significant indicator of poor outcome in cancer including acute myelogenous leukaemia (AML). In addition to its primary drug efflux role, P-gp over-expression may also exert a protective influence on a cell's ability to undergo apoptosis in response to certain stimuli.

A wide range of P-gp inhibitors have been developed for clinical use in an attempt to modulate the MDR phenotype. Zosuquidar Trihydrochloride (Z.3HCL) is a potent and specific third generation P-gp inhibitor and functions in a non-competitive manner.

Z.3HCL was the subject of three phase I clinical trials: two on patients with solid tumours and one on 16 patients with AML. Functional and expression assays were conducted on $CD56^+$ NK cells isolated from patients enrolled on these studies. Safety and efficacy data were also analysed, where available. Z.3HCL was also implemented in a number of *in vitro* assays investigating P-gp expression and function in malignant cells isolated from patients with haematological malignancies: 48 with AML, 75 with CLL, and 16 with MM. Apoptosis assays, utilising normal, patient, and cultured cells were undertaken to investigate the putative role of P-gp in apoptotic modulation.

In the solid tumour trials, *in vitro* assays showed that Z.3HCL infusion was associated with rapid inhibition of P-gp mediated efflux in $CD56^+$ NK cells in 85.2% patients studied. Of the patients enrolled on the AML trial, 11 achieved a complete remission and one a partial remission, with a median survival of 559 (range 38-906) days. Non-haematologic grade 3 and 4 toxicities were observed in four patients. The *in vitro* assays showed that Z.3HCL infusion was associated with rapid inhibition of P-gp mediated efflux in $CD56^+$ NK cells in all 16 patients, and in $CD33^+$ cells from 6/10 patients. The median IC50 for daunorubicin (DNR) using a MTT assay, decreased significantly between

Z.3HCL modulated and unmodulated cells (153 and 247 ng/mL respectively, P=0.01).

In vitro studies showed that 38.2% of samples from AML patients, 61.3% from CLL and 41.2% from MM over-expressed P-gp. Drug sensitisation studies on AML cells showed significantly increased drug sensitivity in P-gp positive co-incubated with Z.3HCL and DNR, which was not observed for P-gp negative cells or for co-incubation with cytarabine. Further studies in CLL patients showed that P-gp function and expression did not correlate with other prognostic indicators such as Binet stage, ZAP-70 expression or IgV_H mutation status.

The intermediately P-gp expressing cell line CEMv showed a significant resistance to ionising radiation compared with CEM cells. Apoptosis assays demonstrated that modulating P-gp with Z.3HCL was able to increase significantly the apoptotic sensitivity above baseline in the highly P-gp expressing CEM-VLB_{100} cells, an effect not seen in the P-gp negative parent CEM cells.

Declaration

The work contained in this thesis is the result of original research carried out by myself under the supervision of Dr. K. Ganeshaguru and Dr. R.G. Wickremasinghe. All sources of information have been specifically acknowledged by means of reference. None of the work contained in this thesis has been used in any previous application for a degree.

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Abbreviations

ABC	ATP Binding Cassette
ALL	Acute Lymphoblastic Leukaemia
AML	Acute Myelogenous Leukaemia
APL	Acute Promyelocytic Leukaemia
ARA-C	cytosine arabinoside (cytarabine)
ATP	Adenosine 5' -triphosphate
ATRA	All Trans Retinoic Acid
BBB	Blood-Brain Barrier
B-CLL	B-cell Chronic Lymphocytic Leukaemia
BCRP	Breast Cancer Resistance Protein
CHF	Congestive Heart Failure
CML	Chronic Myeloid Leukaemia
CsA	Cyclosporine A
DFS	Disease free survival
DISC	Death Inducing Signal Complex
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DNR	Daunorubicin
DXR	Doxorubicin
DXRL	Doxorubicinol
DTT	Dithiothreitol
ECOG	Eastern Co-operative Oncology Group
EDTA	ethylenediaminetetra acetic acid
FACS	Fluorescence-Activated Cell Sorter
FADD	Fas-associated death domain protein

FCCP	carbonylcyanide p-trifluoromethoxyphenylhydrazone
FCS	Foetal Calf Serum
FLU	Fludarabine
FTC	fumitremorgin C
HBSS	Hank's Balanced Salts Solution
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HSC	Haematopoietic stem cell
H_2O	Water
IgV_H	Immunoglobulin Heavy Chain Variable Gene
IKK	IkappaB Kinase
IV	Intravenous
LRP	Lung Resistance Protein
LSC	Leukaemic stem cell
JC-1	tetrachlorotetraethylbenzimidazolylcarbocyanine iodide
MCF	Median Channel Fluorescence
MELP	Melphalan
MGG	May-Grunwald-Giemsa stain
MIT	Mitoxantrone
MNC	Mononuclear cell
MRP	Multidrug Resistance Associated Protein
MTT	3,4,5-dimethylthiazol-2,5-diphenyl tetrazolium bromide
MUGA	Multiple Gated Acquisition scan
NF-κB	Nuclear factor-kappa B
NO	Nitrous oxide
NBD	Nucleotide binding domain

PAGE	Polyacrylamide gel electrophoresis
PARP	Poly-ADP-ribose-polymerase
PBS	Phosphate-buffered saline
P-gp	P-glycoprotein
PI	Propidium Iodide
PI3K	Phosphoinositol-3 kinase
PKC	Protein Kinase C
PMSF	Phenyl methyl sulfonyl fluoride
PS	Phosphatidylserine
PRED	Prednisolone
RBC	Red blood cells
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute (cell culture media)
SAE	Serious Adverse Event
SNP	Single nucleotide polymorphism
SRBC	Sheep red blood cells
T-CLL	T-cell Chronic Lymphocytic Leukaemia
TMD	Transmembrane Domain
VPM	Verapamil
VNC	Vincristine
WBC	White Blood Cell Count
Z.3HCL	Zosuquidar Trihydrochloride
ZAP-70	Zeta-Chain Associated Protein 70

1. Introduction

1.1 General Introduction

Cancers by their very nature are resistant to the death impulse that governs and maintains the regulation of cell number in multicellular organisms[1,2]. For many cancers, and in particular the malignancies that affect haematological tissues, the front-line therapeutic strategy involves the use of cytotoxic agents to arrest and reverse the pathogenic process[3]. The mechanism of action for many of these compounds is the induction of apoptosis, or programmed cell death, in the tumour cells whilst leaving normal tissues relatively unharmed[4].

The real-world application of chemotherapeutic agents is fraught with a multitude of complexities that undermine their efficacy in the treatment of cancer. The inherent metabolic heterogeneity of patients affects the clearance rates of the drugs, non-specificity of the active compounds leads to systemic toxicity, and the innate adaptability of tumour cells gives rise to drug resistance[5].

Chemotherapy drugs are toxic to a wide spectrum of cell types and accordingly tend to have a narrow therapeutic window. Apoptosis is a tightly controlled mechanism of programmed cell death requiring the activation of appropriate cellular signalling mechanisms in order to commit the cell to this auto-destructive path[6]. Processes that cause perturbations in the apoptotic signal being delivered by the drug to the target cell will render that signal impotent, allowing the cell to live and ultimately leading to chemotherapeutic failure. In addition, sub-lethal doses of the cytotoxic drug will not only apply selective pressure in favour of cells with resistance mechanisms, but will compound the problem by inducing further upregulation of those mechanisms[7].

1.2 Apoptosis and Cancer

Cancers can be characterised as the pathological accumulation of abnormal cells which invade, subvert and supplant the host tissues[8]. A clonal expansion triggered by a genetic or epigenetic aberration[9,10], the accretion of neoplastic cells is driven by two important factors: proliferation and immortality, the latter principally through the evasion of apoptosis. These two hallmarks of malignancy result from a breakdown in the tightly regulated mechanisms of cellular homeostasis which normally lead to differentiation and apoptosis - both of these being of paramount importance to the understanding of why cancers occur and for the development of curative strategies[11].

Cellular differentiation involves the attainment from an intrinsically proliferative and functionally immature precursor cell to a functionally mature, usually non-proliferative cell type. Oncogenic processes that arrest a cell at its precursor state will give rise to a clonal expansion of sub-functional cells which may eventually supplant the normal functioning population of cells, leading to disease[12]. Therapy that can force a tumour cell down its differentiation pathway, such as all trans retinoic acid (ATRA), which is used in the treatment of Acute promyelocytic leukaemia (APL), can make a significant contribution to achieving a cure[13].

Apoptosis results from a series of intracellular mechanisms which when activated causes target cells to undergo what is commonly known as programmed cell death[14-16]. This type of cell death is distinct from necrotic or catastrophic cell death in that it is a strictly regulated and effector driven cascade of events leading to intracellular and nuclear degradation while maintaining

plasma membrane integrity[17]. This ensures that there is no release of lytic agents from the cell's endosomes that may cause damage to the local tissues and trigger an inflammatory response. At the culmination of the apoptotic process the cell's DNA has been digested and condensed, the plasma membrane has become contracted and highly blebbed and the cell forms what is known as an apoptotic body, which can be safely removed by macrophages or absorbed by surrounding cells via phagocytosis[18].

The main effectors of the apoptosis pathway are a set of homologous cysteine proteases collectively known as the caspases (from *cysteinyl-aspartate-cleaving proteases*). These are synthesized as enzymically inert zymogens that upon activation cleave target substrates (including other caspase precursors) thus generating a signal amplifying cascade[19]. There are thought to be about 12 caspases involved in apoptosis, and essentially, caspase 9 and caspase 8 can be thought of as initiator molecules while caspase 3, caspase 6 and caspase 7 are the proteolytic effectors[20].

The apoptotic pathway can be triggered by a variety of stimuli: DNA damage, growth factor deprivation, ligation of the tumour necrosis factor receptor (TNF-R) family and the action of cytotoxic drugs. The initiation of apoptosis is modulated by families of pro- and anti apoptotic proteins, among the most prominent being the essential pro-apoptotic checkpoint molecule, p53, the anti-apoptotic Bcl-2, Fas-associated protein with death domain (FADD), and the inhibitor of apoptosis proteins (IAPs)[21-23].

The evasion of apoptotic signalling is one of the key events in the progression of

malignant disease, and it is the aim that many chemotherapeutic agents may selectively restore apoptotic sensitivity to the tumour cells[24].

1.3 Classes of Anti-cancer Drugs

Many cytotoxic agents in use are derived from natural sources such as tree bark (Taxol from Yew), microorganisms (Daunorubicin) and flowering plants (vincristine from periwinkle). Most enter the cell via passive diffusion across the plasma membrane, but some are transported via receptors (such as the folate analogue methotrexate[25]) or are endocytically internalized via pinocytosis (such as some classes of immunotoxins[26]). Generally, their defining feature is that they are more toxic to cancer cells than to normal cells; this is often because of the higher proliferation rate of malignant cells.

Once they are in the cell, the drugs have to travel to their site of action to achieve their cytotoxic effect, which is usually the nucleus since virtually all affect DNA synthesis or cell division. The anthracyclines, such as daunorubicin are intercalating agents that integrate into the DNA helix. They disrupt DNA replication during cell division and inhibit the topoisomerase enzymes resulting in cell cycle arrest and cell death via tumour suppressor checkpoint proteins such as p53.

The majority of commonly used chemotherapeutics can be divided into functional classes such as alkylating agents, anti-metabolites, plant alkaloids and other naturally derived agents, anti-tumour antibiotics, and hormones.

The alkylating agents include the nitrogen mustard analogues, such as chlorambucil and melphalan, and the alkyl sulphonates such as busulfan[27]. The anti-metabolites include folic acid analogues (methotrexate), purine analogues (mercaptopurine), and pyrimidine analogues (cytarabine)[28,29]. The plant alkaloids and other agents include the vinca alkaloids (vincristine), podophyllotoxins (etoposide), and taxanes (paclitaxel)[30]. The anti-tumour antibiotics include anthracyclines such as mitoxantrone and daunorubicin, as previously discussed[31].

Hormones can also be used as chemotherapeutic agents such as the steroid dexamethasone, which can inhibit the growth of some tumours, and hormone blocking agents, used in sex-hormone driven malignancies, such as finasteride and tamoxifen for prostate and ovarian cancers respectively[32]. Other anti-cancer agents include the platinum compounds, such as cisplatin, and monoclonal antibodies such as rituximab, which binds to the CD20 antigen and is used to treat certain B cell malignancies[33].

1.4 Multidrug Resistance

Multidrug resistance (MDR) is an innate or acquired characteristic describing a number of cellular mechanisms that conspire to render a cell impervious to a range of structurally and functionally disparate cytotoxic agents[34]. A hallmark of the acquired trait is the ability of a single applied cytotoxin to engender a broad spectrum resistance phenotype[35]. The innate expression of MDR is cell and tissue specific, and tumours arising from these cellular sources will be more likely to display the MDR phenotype[36]. Tumours expressing or acquiring the

MDR phenotype have been shown to be harder to treat with chemotherapy and correlate with a less optimistic prognosis[37-39].

The best studied MDR mechanism is the expression of membrane efflux pumps. Their basic mode of action is to nullify the toxicity of a wide range of unrelated anticancer drugs by increasing their cellular efflux across the plasma membrane, resulting in reduced drug accumulation at the intracellular site of action[40]. Energy in the form of ATP hydrolysis is required for their operation, and they can actively remove compounds against a concentration gradient[41]. There are several different members of drug efflux pumps in this class, the best studied and possibly the most clinically significant being the P-glycoprotein (P-gp)[42].

Other MDR mechanisms include: detoxification mechanisms (glutathione transferase, *metallothionein and selenium* dependent glutathione peroxidase), alteration of drug target site by mutation (e.g., BCR-ABL tyrosine kinase resistance to Imatinib Mesylate), enhanced DNA repair, lowered drug activation, and cell cycle and apoptosis mediated resistance (BCL-2, p53)[43-45]. There is one family of membrane associated efflux pumps that have become almost synonymous with the classical MDR mechanism of reduced drug accumulation, and they are the ABC Transporters[46].

1.5 ABC Transporters

The ATP-binding cassette (ABC) transporters are a superfamily of highly conserved membrane proteins responsible for the transport of a variety of molecules across the plasma membrane, as well as intracellular membranes[47]. There are currently 49 known human ABC transporters, classified into seven subfamilies, ABCA, ABCB, ABCC, ABCD, ABCE, ABCF and ABCG[48,49]. They use energy from the hydrolysis of ATP to translocate a target substrate molecule across their associated membrane from one compartment to another, typically against a concentration gradient[50].

The characteristic functional structure of ABC transporters is that of two homologous halves, joined by a linker region, each with a transmembrane domain (TMD) and a nucleotide binding domain (NBD)[51] (Figure1.1). Each TMD typically comprises of 6-11 membrane spanning α-helices which determine the substrate specificity. There are some ABC proteins, however, that comprise of a so called half-transporter; consisting of only a single TMD and NBD, they are thought to operate functionally as a homodimer, or even in some cases as a heterodimer[47,52].

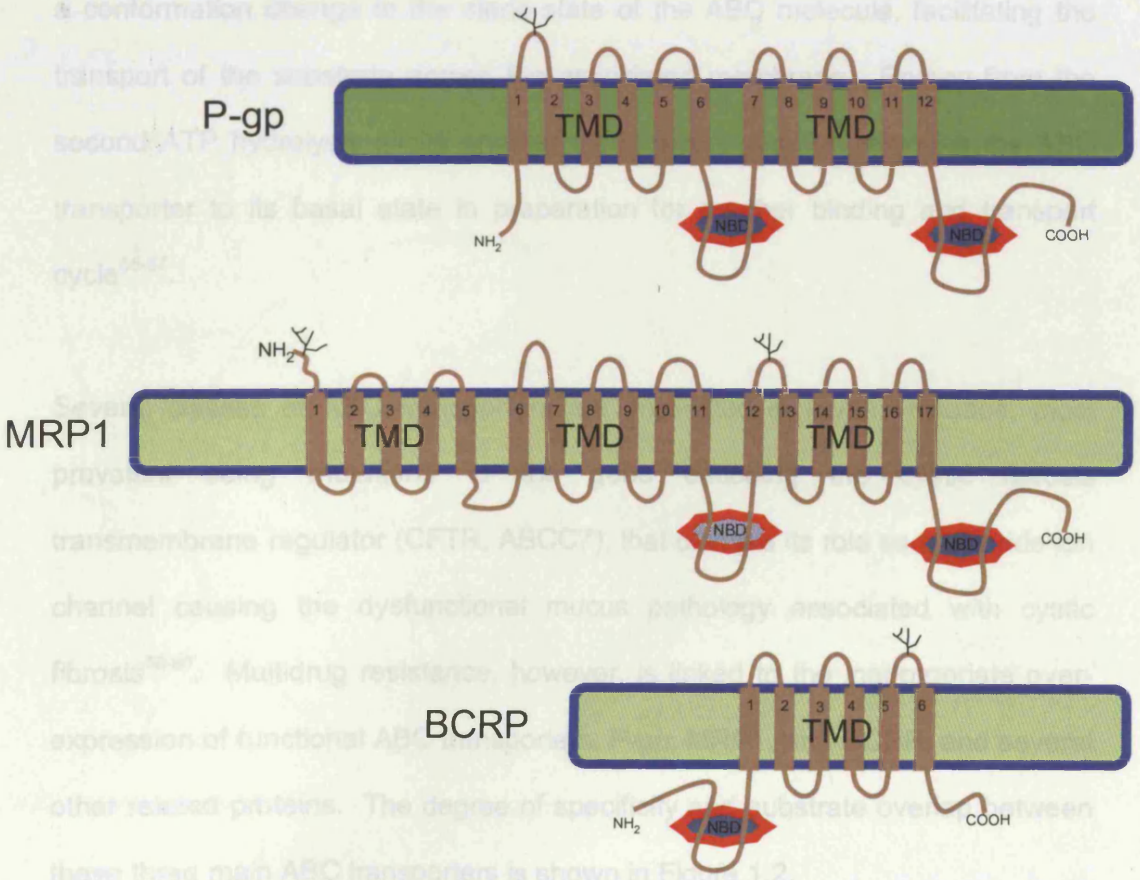
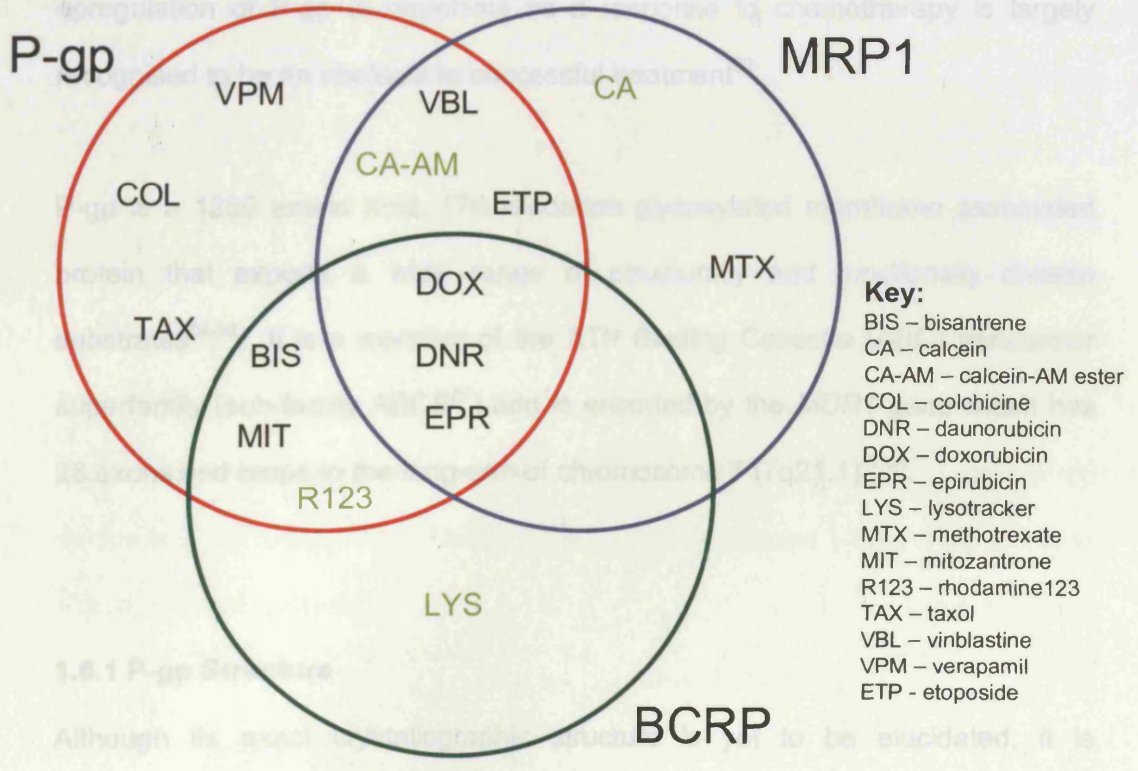


Figure 1.1 Basic structure of three ABC transporters implicated in MDR: P-glycoprotein (P-gp), Multidrug resistance associated protein (MRP1), and Breast cancer resistance protein (BCRP). TMD – Transmembrane domain; NBD – Nucleotide binding domain.

The NBD's are located in the cytoplasm and contain three conserved domains: the Walker A and B domains and a signature C motif, which is found upstream of the Walker B site[53,54]. The NBD's translate and transfer energy liberated from the catalytic hydrolysis of ATP to ADP into the active transport of the substrate. Typically, two molecules of ATP are bound at the NBD's and the target substrate is bound by one of the TMD's. Energy from one of the hydrolysing ATP's causes

a conformation change in the steric state of the ABC molecule, facilitating the transport of the substrate across the associated membrane. Energy from the second ATP hydrolysis elicits another steric reconfiguration returning the ABC transporter to its basal state in preparation for another binding and transport cycle[55-57].

Several classes of ABC transporters are implicated in human disease, most prevalent being mutations to the gene encoding the cystic fibrosis transmembrane regulator (CFTR, ABCC7), that disrupts its role as a chloride ion channel causing the dysfunctional mucus pathology associated with cystic fibrosis[58-60]. Multidrug resistance, however, is linked to the inappropriate over-expression of functional ABC transporters: P-gp, MRP1, and BCRP, and several other related proteins. The degree of specificity and substrate overlap between these three main ABC transporters is shown in Figure 1.2.



Adapted from Litman *et al*, 2001

Figure 1.2 Venn diagram illustrating substrate overlap between the three main MDR transporters, P-gp, MRP1 and BCRP. The compounds coloured green are efflux dyes commonly used for studying MDR function.

1.6 P-glycoprotein

ABCB1, MDR1, P-gp, PGY1, GP170

The permeability glycoprotein (P-gp) is the best known and most extensively studied contributor to the MDR phenotype. It was first described by Juliano and Ling in 1976 from work involving colchicine resistant Chinese Hamster Ovary cells[61], although studies by Dano in 1973 identified active transport of daunomycin in Ehrlich tumour cells[62]. Inappropriate over-expression and

upregulation of P-gp in neoplasia as a response to chemotherapy is largely recognised to be an obstacle to successful treatment[63].

P-gp is a 1280 amino acid, 170 kilodalton glycosylated membrane associated protein that exports a wide range of structurally and functionally diverse substrates[64-66]. It is a member of the ATP Binding Cassette (ABC) transporter superfamily (sub-family ABCB[67]) and is encoded by the *MDR1* gene which has 28 exons and maps to the long-arm of chromosome 7 (7q21.1)[47,68].

1.6.1 P-gp Structure

Although its exact crystallographic structure is yet to be elucidated, it is commonly believed that the P-gp molecule has a typical ABC transporter configuration, consisting of two homologous halves of about 610 amino acids, each with six α–helix transmembrane domains and an ATP binding motif (Figure 1.1). The two homologous sections are joined by a flexible linker region of about 60 amino acids, and each TMD has four intracellular loops, each made up of 50 amino acids, connecting the α–helices on the cytoplasmic side of the membrane[69]. It is envisioned that the P-gp molecule resides in the lipid phase of the associated membrane and folds spatially to form a pore-like structure with a symmetry analogous to a heterodimer, but a single protein coded by a single gene (Figure 1.3).

The P-gp molecule is glycosylated at three N-glycosylation sites in the first extracellular loop. Mutational and deletion analysis shows that these

glycosylations are required, not for efflux function, but for the correct trafficking of the P-gp protein to the plasma membrane[70,71].

Studies using photo affinity substrate analogues to map P-gp substrate binding interactions have shown that different substrates have different, but overlapping binding sites. All the areas implicated exist within the α-helix transmembrane domains[72]. Further evidence for the location of the substrate binding domain came from site directed mutagenesis studies that showed that mutant P-gp molecules that lacked both NBD's (and so only consisted of the TMD's) could still bind drug substrates[73].

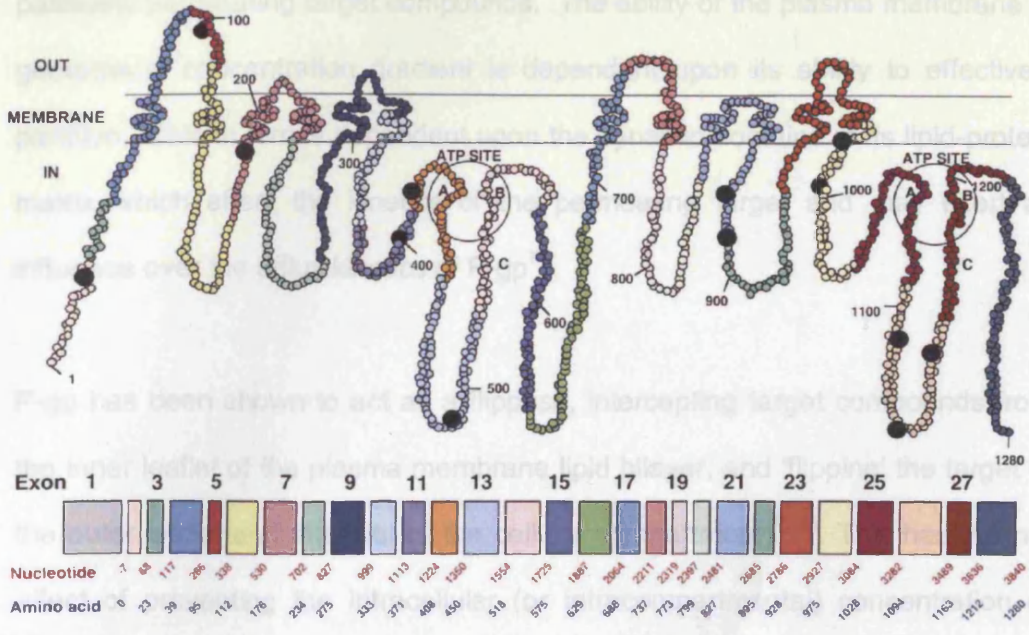


Figure 1.3. Structural diagram of P-gp. The transdomain regions span the plasma membrane and the folded structure may be envisioned as pore-like. Also shown is the exon sequence relating to amino acid position. From Ambudkar *et al*, 2003

1.6.2 Mode of Action of P-gp

The plasma membrane forms a differentially permeable partition between the interior of the cell and the functional milieu of its tissue environment. The plasma membrane is a fluid lipid-protein matrix into which is embedded a multitude of receptors, effectors and signalling components all involved in dynamic interactions necessary to the function and continuing survival of the cell. P-gp interacts with, responds to, and exerts an influence over its immediate lipid-membrane environment.

P-gp is concerned with affecting the net permeability of the plasma membrane, primarily through the active effluxing against a concentration gradient of passively permeating target compounds. The ability of the plasma membrane to generate a concentration gradient is dependent upon its ability to effectively partition. This in turn is dependent upon the dynamic domains of its lipid-protein matrix which affect the kinetics of the permeating target and may exert an influence over the efflux kinetics of P-gp[74].

P-gp has been shown to act as a flippase, intercepting target compounds from the inner leaflet of the plasma membrane lipid bilayer, and 'flipping' the target to the outer leaflet and thus out of the cell (or compartment)[75,76]. This has the net effect of preventing the intracellular (or intracompartmental) concentration of administered cytotoxic drugs reaching an effective therapeutic level.

1.6.3 P-gp Isoforms and Homologues

There are two P-gp isoforms in humans (MDR1 and MDR3)[77] and three in rodents (mdr1a, mdr1b and mdr1c)[78]. MDR3 (ABCB4 encoded by the *MDR3* gene, mapping to 7q21.1) may have a physiological role in the liver translocating phosphatidylcholine into bile, and is most strongly expressed on the hepatocyte canalicular membranes[79]. Mutations arising in MDR3 have been implicated in an increased susceptibility to the bile disorders Familial Intrahepatic Cholestasis and Intrahepatic Cholestasis of Pregnancy[80,81].

Although MDR3 has some substrate overlap with P-gp it has not been shown to be significantly involved in drug resistance. However, some studies have reported an increase in drug uptake by MDR3 expressing leukaemia cells treated with cyclosporine[82-84]. MDR3 is homologous to the murine mdr2[85]

Table 1.1 Summary of tissue sites of physiological P-gp expression.

Sites of Elevated P-gp Expression
Liver Biliary canalicular membrane
Kidney Brush border of renal proximal tubules
GI Tract Apical surface of intestinal mucosal cells
Endocrine System Adrenal Gland
Blood - Tissue Barriers *Capillary endothelial cells of:* Brain Testes Ovaries Placenta
Haematological Cells CD34+ Stem Cells CD56+ Natural Killer (NK) CD8+ T-cells

Two proteins closely related to P-gp have recently been described. One is the recently characterised ABCB5 P-gp which is an 812 amino acid protein encoded on chromosome 7p21-15.3 and whose expression is lineage restricted to the plasma membrane of $CD133^+$ human epidermal progenitor cells. Its physiological function appears to be concerned with the control of progenitor cell fusion to form multinucleate constructs through regulating membrane potential depolarisation[86]. ABCB5 P-gp has been reported to be able to efflux R123, but no role in contributing to MDR has been elucidated.

The second is the 'Sister of P-glycoprotein' (sPgp, ABCB11), which is a specific bile acid transporter expressed only in the liver canalicular membrane[87,88]. Its gene map locus is 2q24, and mutations to this gene are associated with progressive familial intrahepatic cholestasis; however, sPgp is not thought to play a role in MDR.

1.6.4 Physiological Role of P-gp

P-gp is constitutively expressed by three basic cell types: tissue barrier, excretory surface, and haematological cells[89]. It is found in high levels at the apical surface of cells typically associated with transport leading to a proposed primary role as a cellular detoxifier through the active secretion of xenobiotics and natural toxins[90]. This theory is supported through murine *mdr* gene knockout studies which show a role for P-gp in drug absorption, disposition, elimination, and detoxification pathways[72,91,92].

There is evidence for several secondary regulatory roles. The activity of P-gp is highly influenced by the lipid component of the membrane it is embedded. Since the make-up of the lipid phase of cells is strongly dependant upon the cell type, it is reasonable to assume that the determinants of physiological P-gp function are likely to be cell type specific[74].

Highest normal physiological expression of P-gp is to be found on the apical surface of gut epithelia[93], liver cells[94], kidney tubules[95,96] and blood-tissue barriers (brain and testes)[97,98]. Elevated expression is also found on haematopoietic stem cells ($CD34^+$)[99-103], Natural killer cells (NK), and T and B lymphocytes[104]. Intestinal expression of P-gp can have a severe limiting affect upon the bioavailability of substrate compounds ingested via the oral route and so restricting the efficiency of orally administered drugs (Table 1.1)[105,106].

NK cells have highest expression of P-gp of all the leukocytes suggesting that it may play a role in it's cytotoxic function[107]. Moderate to high expression of P-gp is also found in $CD8^+$ T-cells, which are also involved in cell mediated cytotoxicity. $CD4^+$ T-cells and B-cells, which are not cytotoxic effector cells, show relatively lower P-gp expression.

There is evidence to suggest that P-gp may have a role in protecting against some caspase-mediated apoptosis, including apoptosis induced by serum starvation and via ligation of Fas[90,108,109]. The over expression of P-gp by a cell has been shown to have an effect on cholesterol metabolism and membrane redistribution[90,110]. There is a possibility that these membrane reorganization effects may contribute to the reduction in sensitivity to certain forms of pro-

apoptotic stimuli. Another little researched function is that P-gp (and MRP1) appear to have a role in the migration of dendritic cells from the skin via afferent lymphatic vessels[111,112].

P-gp expression is associated with regulating the activity of volume-activated chloride ion (Cl^-) channels, which operate as a defensive response to increased cell swelling. There is evidence that phosphorylation (possibly by protein kinase C (PKC) or protein kinase A (PKA)) of the linker region that join the two homologous halves of P-gp may be involved in regulating Cl^- conductance. This is analogous to the R-region of CFTR, since the two transporters have similar domain structure[113,114].

In patients with cystic fibrosis, it has been observed that there is a concomitant upregulation of the expression of P-gp in the intestine in the absence of CFTR, and that this may be a compensatory effect reflecting the complementary pattern of expression shown by the two ABC proteins[115].

There appears to be a background ATPase activity associated with P-gp, independent of drug binding and efflux. It was initially thought to be stimulated by the binding and transmembrane movement of molecules such as small lipid molecules[76] or cholesterol[116], but a subsequent study has demonstrated evidence for a basal level of ATPase activity even in the absence of any substrate binding[117].

1.6.5 P-gp Substrates

P-gp has a broad range of structurally diverse substrates which gives rise to the phenomenon of MDR, whereby the resistance mechanism(s) engendered by a single cytotoxic entity is effective against a spectrum of structurally and functionally unrelated compounds[118]. Generally, the target substrates for efflux are the natural or semi-synthetic drugs, which are neutral or cationic hydrophobic or amphipathic molecules that achieve partition equilibrium via passive diffusion across the plasma membrane[119]. These compounds are not dependent on rate-limiting influx mechanisms such as receptors or ion pores (necessary for the transport of hydrophilic molecules) so the only way to reduce their intracellular accumulation is either to remove them from the cytoplasm or to intercept them in the membrane and flip them back into the intercellular medium[72].

P-gp has at least two allosterically linked substrate binding sites which are associated with the transmembrane domains (TMD's). These in turn are allosterically linked to the nucleotide binding domains (NBD's), and together perform as a unit that is engaged in an ATP powered cycle of substrate binding, transmembrane transport and renewal. P-gp has a broad range of structurally diverse substrates including the vinca alkaloids, anthracyclines, epipodophyllotoxins, taxanes, ATRA, Gleevec (STI571), HIV protease inhibitors, steroid hormones, anti-epileptic drugs, and fluorescent dyes (Table 1.2).

Table 1.2. Examples of chemotherapy drugs affected by P-gp over-expression in the treatment of cancer and HIV.

Drug Substrates for P-gp		
Class	**Example**	**Action**
Alkaloid	Colchicine	Spindle Inhibitor
Anthracyclins	Daunorubicin Doxorubicin Mitoxantrone	Intercalation agent / Topoisomerase II Inhibitor
Vinca alkaloids	Vincristine Vinblastine	Spindle inhibitor
Epipodophyllotoxins	Etoposide	Mitotic inhibitor/Topo-isomerase II Inhibitor
Taxanes	Paclitaxel Docetaxel	Microtubule stabilizer
Targeted Therapies	Gleevec (STI571)	BCR-ABL Tyrosine Kinase Inhibitor
Vitamin Analogues	ATRA	Differentiation agent
Steroid hormones	Dexamethasone Prednisone	Immunomodulators
HIV Drugs	Saquinavir	Protease inhibitors

1.6.6 P-gp and CYP3A4

P-gp exhibits a considerable substrate overlap with the CYP3A4 (cytochrome p450) enzyme, which plays a central role in the metabolism and excretion of many of the drugs and xenobiotics implicated as substrates[120-122]. P-gp and CYP3A4 are co-expressed in the liver and intestine and their expression can be co-ordinately regulated by the steroid and xenobiotic receptor (SXR)[123,124].

1.6.7 P-gp Polymorphisms

There are several known MDR1 gene polymorphisms that occur in the human population[125]. Currently, over the 28 exons, 28 single nucleotide polymorphisms (SNP) have been identified at 27 positions[126]. One of the best studied SNP's is the C3435T substitution on exon 26, which has been shown in some studies to result in a lower intestinal P-gp expression. It is interesting to note in the case of the C3435T substitution that although there is a DNA base change between the polymorphic genotypes, which appear to produce some degree of functional variability, the SNP does not result in an amino-acid change. The inherent redundancy in the generic coding sequence means that both codons involved translate the same amino-acid. The functional variability may occur because of some difference between the polymorphic mRNA transcripts, such as unequal processing efficiencies. Another possibility is that the C3435T is linked to another gene which is co-inherited and which does have the ability to affect P-gp expression or function[72].

The resultant deficiency in P-gp expression has been shown to be associated with a greater susceptibility to the development of ulcerative colitis in some populations. This demonstrates the important role played by P-gp as part of the defence mechanism against intestinal bacteria and toxins, and that pathologies can occur when this barrier function endowed by P-gp is compromised[127-129].

It has also been observed, however, that several polymorphisms, including the C3435T genotype do not affect the ability of that P-gp to exclude fluorescent dyes, such as rhodamine123 (R123) from cells expressing it, implying that the functional capability of the expressed P-gp protein is not impaired[130]. Hitzl *et al*

showed $CD56^+$ cells isolated from C3435T homozygous (TT) and heterozygous carrier (CT) did show a significantly decreased (TT) and intermediately decreased (CT) P-gp mediated R123 efflux compared to normal (CC) individuals[131]. No distinction was given as to whether this was a result of reduced function or reduced expression.

The exon 26 genotype has been shown to be an independent prognostic indicator for acute persistent rejection in lung transplants. It is thought that because many of the immunosuppressive drugs necessary for the prevention of rejection are P-gp substrates, the drug distribution phenotype of the patient may play a role in rejection status. The wild-type CC genotype was found to be associated with a significantly higher risk of organ rejection than the TT polymorphism. The increased P-gp function associated with the CC genotype is thought to contribute to rejection by lowering the efficacy of the immunosuppressive drugs by altering their tissue distribution or by altering T-cell resistance in some way[132].

However, a meta-analysis by Chowbay, *et al* of studies investigating C3435T influence on P-gp expression and digoxin pharmacokinetics ultimately found no causal link between the polymorphism and the attributed effects. They suggest that the previously observed results may be due to a more complex interaction between SNP haplotypes and/or other polygenic traits, which in combination may influence expression and distribution. The conclusion advocated that further studies should avoid focusing on single SNPs in order to avoid spurious pharmacogenetic associations[133-135].

Another polymorphism associated with a clinical significance is the G2677T SNP in exon 21. Renal transplant patients homozygous for this substitution have been shown to exhibit a 40% increased dose requirement compared with the wild type population for the oral immunosuppressant tacrolimus. Furthermore, genotype analysis by haplotype analysis of the *MDR1* gene reliably predicted which patients would require optimum dose modification[136].

1.6.8 Regulation of P-gp Expression

The expression of P-gp can be regulated at the DNA, mRNA, or protein levels, and the predominate control modality varies between cell types[137-139]. The homeostatic signalling feedback pathways that determine P-gp expression and function have been difficult to elucidate due to the varying levels of expression and function in the range of different cell types involved.

The protein activation regulator Protein Kinase C (PKC)[140-146], the cell cycle checkpoint protein p53[147-155] and the nuclear transcription factor NF-κB[156-160] have all been implicated with at least some level of control over P-gp expression levels. Signalling via the phosphoinositol-3 kinase (PI3K) pathway has also been shown to upregulate P-gp expression[161]. In enterocytes, P-gp expression and function can be upregulated by NO and pro-inflammatory cytokines in a PI3K dependent manner. It can be hypothesised that this effect may be part of an adaptive mechanism to limit epithelial injury during response to inflammatory conditions[162].

1.6.8.1 PKC

PKC describes a group of at least 12 calcium and phospholipid dependent isoenzymes, which exhibit their mode of action by phosphorylating serine and threonine residues of target proteins. The 12 isoenzymes are divided into three subgroups: conventional (calcium dependent), novel (calcium independent) and atypical. Generally, 'PKC' usually refers to the PKCα isoenzyme, which is a member of the conventional subgroup. Through this modulatory phosphorylation action PKC plays a pivotal role in cell signal transduction and the regulation of protein and enzyme activity[144,163].

It has been observed that in the creation of many MDR cell lines that there is a concomitant increase in PKC levels, and there is evidence to suggest that a complex relationship exists between P-gp expression, function and PKC. This interaction appears to exist both at the gene level, through PKC mediated regulation of MDR1 transcription, and at the protein level through PKC phosphorylation of key P-gp serine residues. The role PKC plays in the regulation of MDR1 at the transcription level is poorly understood and evidence for it is based mainly on correlative expression levels in cell lines. Higher levels of PKC do correlate with increased P-gp expression, but again this is complicated by factors such as cell type, environmental conditions, time scale and the interaction of PKC isoenzymes under consideration[164,165].

Studies examining the post-translational modification of P-gp by PKC have shown that positioned throughout its primary sequence P-gp possesses up to 35 potential sites for PKC phosphorylation. However, of these only three serine residues have been recognised to be phosphorylated by PKC *in vivo:* residues

661, 667 and 671, which reside in the inter-TMD linker region. It is postulated that phosphorylation of these sites by PKC can regulate the activity of P-gp in several ways: phosphorylation of serine 671 has been linked to a stimulation in the activity of P-gp's ATPase, so increasing its ability to act as drug transporter[166]. Phosphorylation of residue 667 has been shown to correlate with the suppression of P-gp associated volume-activated chloride channel activity. Cell volume increase appears to lead to a pattern of phosphorylation that decreases ATPase activity and activates the chloride channel. It would thus appear that PKC represents a flexible mechanism by which the cell may respond adaptively to a range of stimulatory circumstances[114].

It is interesting to note that even though there are three serine residues in the P-gp linker region that are PKC phosphorylation sites it has been shown that deletion mutants lacking these sites exhibit normal MDR efflux characteristics[167]. This would suggest that phosphorylation activity at this region is involved with the regulation of some primary efflux-function independent mechanism, or at least that the action of PKC is a cell type and condition dependent mechanism. Other studies, however, have reinforced the link between PKC and P-gp function, demonstrating that elevated levels of PKC and P-gp are concomitant in MDR cell models[168], and that PKC inhibitors, such as PKC412 have been shown to reduce MDR activity in P-gp expressing cells[143].

1.6.8.2 P53

The tumour repressor protein p53 can modulate the expression of P-gp by repressing the transcription of the MDR1 gene. This is achieved by direct

binding of p53, in a novel tetrameric conformation, to a proximal binding element within the MDR1 promoter[169,170]. Certain p53 mutations can cause a functional *volte-face* so that it becomes an inducer of P-gp, though probably not through a direct promoter interaction[171]. The loss of p53, either through deletion or through functional mutation, is considered to be one of the key oncogenic cellular changes and has been shown to be correlated with P-gp expression[151,153,172].

1.6.8.3 NF-κB

NF-κB, a critical cellular mediator of immune and inflammatory response, has been shown to be able to upregulate P-gp expression in kidney proximal tubule cells as part of a protective mechanism in response to the generation of reactive oxygen species (ROS)[156]. It is thought that certain forms of cellular insult such as cadmium poisoning, ionizing radiation or some other carcinogen can cause the generation of the highly reactive and damaging ROS, which in turn trigger upregulation of NF-κB via activation of the I-kappa B kinases (IKK). The IKK kinases promote NF-κB mediated transactivation by the phosphorylation of its inhibitor IκB, which flags IκB for ubiquination and subsequent degradation via the 26S proteasome[173,174]. IκB is constitutively bound to NF-κB and prevents it entering the nucleus, but once IκB is removed, NF-κB translocates to the nucleus and binds to its target gene promoters. One of the proteins to be affected is *MDR1* and upregulation of its expression is initiated[160].

1.6.8.4 Constitutive Expression

The MDR1 promoter region has been shown to contain Sp1 binding motifs and so called Y-boxes (CCAAT elements), which are essential for basal expression. The Y-box sequence is recognised and bound by the nuclear transcription factor NF-Y, which is a complex of three sub-units, A, B, and C. Inhibiting NF-Y with agents, such as the anti-tumour agent HMN214 (via its metabolite HMN-176), has been shown to actively down regulate expression of P-gp while also imparting a chemotoxic function[175,176].

1.6.8.5 St John's Wort

St. John's wort is a commonly used and apparently effective herbal antidepressant which has been shown to be an inducer of P-gp (and CYP3A4) and is therefore contraindicated for use in conjunction with cancer or HIV chemotherapy[177-179].

1.6.9 P-gp Role in Apoptosis

Previous studies suggest a non-drug efflux role for P-gp in cellular resistance to caspase mediated apoptosis[108,109,180,181]. However, P-gp expression in HL-60 cells selected to overexpress P-gp was not found to correlate with apoptotic sensitivity when apoptosis was induced by curcumin or UVC[182].

The apoptosis preventing mechanism involved is unclear, but one hypothesis proposed involves the P-gp mediated transmembrane trafficking of sphingomyelin, thus affecting endogenous levels of ceramide, which has been shown to be a potent trigger of apoptosis in cell lines[183,184]. P-gp has also been

shown to alter intracellular calcium ion (Ca^{2+}) levels, and cytoplasmic pH, both of which contribute to the cell's response to apoptotic stimuli[185,186]. P-gp could also change cytoplasmic concentrations of other, as yet unidentified apoptotic factors. P-gp has also been shown to inhibit FAS and other death domain activation of Caspase 8[187]. Molecules involved in the control of apoptosis such as p53 and NF-κB have been shown to alter P-gp expression in certain circumstances[147,149,150,154,157-159,171,188]. The interaction of P-gp with several of these cell growth and apoptotic control factors is summarised in Figure 1.4.

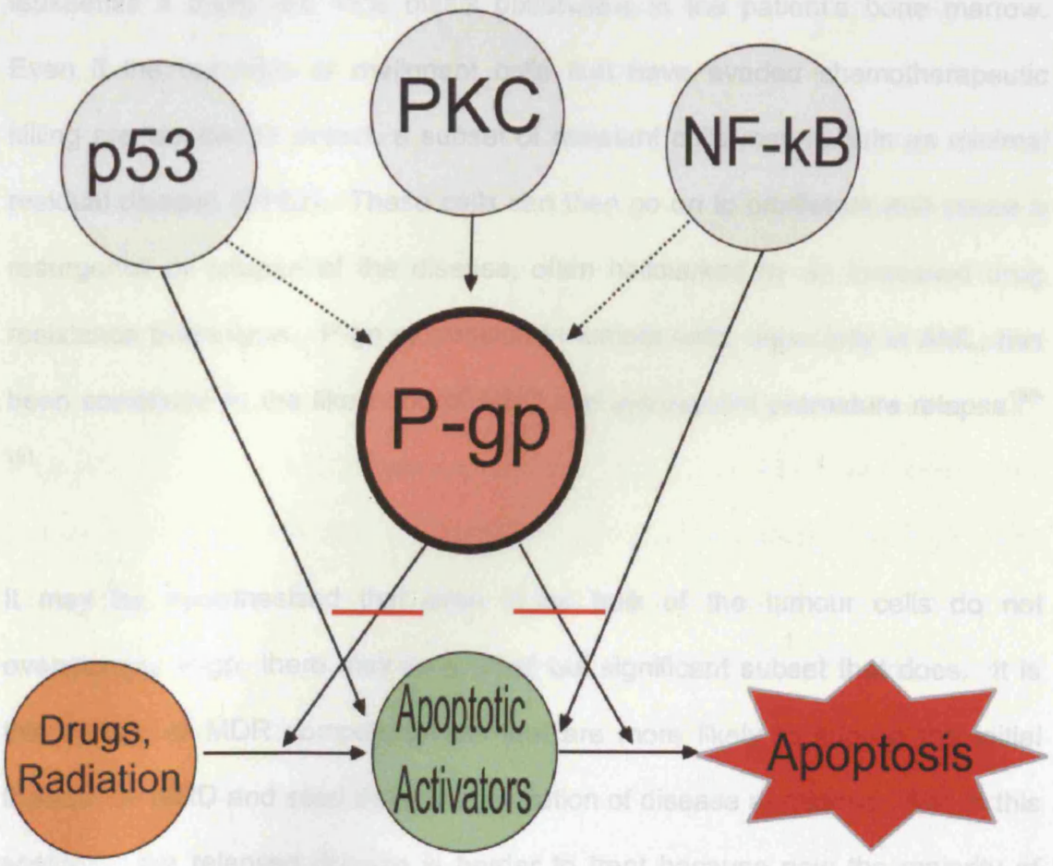


Figure 1.4 P-gp and Apoptosis. Simplified diagram demonstrating factors affecting P-gp expression and the hypothetical influence of P-gp on apoptotic pathways.

1.6.10 Contribution to Minimal Residual Disease

Morphological remission is considered to have been achieved in patients with leukaemia if there are <5% blasts observable in the patient's bone marrow. Even if the numbers of malignant cells that have evaded chemotherapeutic killing are too low to detect, a subset of resistant cells may remain as minimal residual disease (MRD). These cells can then go on to proliferate and cause a resurgence or relapse of the disease, often hallmarked by an increased drug resistance phenotype. P-gp expression in tumour cells, especially in AML, has been correlated to the likelihood of MRD and subsequent premature relapse[189-191].

It may be hypothesised that even if the bulk of the tumour cells do not overexpress P-gp, there may be a small but significant subset that does. It is this subset of MDR competent cells that are more likely to survive the initial therapy as MRD and seed the next generation of disease at relapse. And in this scenario, the relapsed disease is harder to treat because now the majority of tumour cells, like their MRD progenitors, are P-gp expressing. This is especially problematic if combined with another resistance marker[45].

1.6.11 Immunological Resistance

In P-gp over expressing cell line models it has been observed that in addition to the drug pump function of cellular protection, an additional level of resistance to complement mediated cytotoxicity (CMC) is generated[192]. Complement fixation is primarily an anti-microbial defence mechanism which utilizes a cascade model of inactive subunits to generate a membrane perforating pore, (membrane attack

complex), which results in cell lysis. Complement is often triggered by the process of opsonization, which is essentially the binding of antibody to the target cell. The Fc portion of the bound antibody recruits and initiates the complement fixing cascade[193,194].

CMC may play a role in the body's fight against cancer, and immunological therapies that utilize it may be an alternative route to treating tumours with classical MDR, so P-gp engendered resistance to CMC would seem a logical component of the tumour cell's MDR armamentarium. The mechanism whereby P-gp could bestow CMC resistance is unclear, but it has been hypothesised that the concomitant increase in intracellular pH that accompanies P-gp over-expression may contribute to CMC resistance, as well as to other phenomena associated with P-gp, such as decreased apoptotic sensitivity[195], which are not adequately explained by its primary efflux function[185,196].

1.6.12 Factors Associated with P-gp Expression in Leukaemia

P-gp over-expression in haematological malignancies has been widely recognised as an adverse prognostic factor. In AML, P-gp over-expression has been shown to be associated with unfavourable cytogenetic karyotypes, the AML M1 FAB subtype, and increasing age, all of which are indicators of poor response. The association between advancing age and increased P-gp expression in AML, in combination with a greater likelihood of adverse cytogenetic features, lead some to label AML in the elderly (>50 years) as a distinct biologic subgroup associated with a significantly poorer outcome than younger patients[197-199].

The MDR phenotype associated with P-gp over-expression has also been shown to be frequent in myelodysplastic syndrome and in the blast crisis of chronic myeloid leukaemia (CML), both of which are diseases with a poor prognosis[200]. In the latter, P-gp expression may well compound the treatment failure due to imatinib mesylate resistance, since it is an avid substrate for drug efflux in cell line models[201].

In acute lymphoblastic leukaemia (ALL), P-gp expression is again associated with other poor prognosis markers, especially BCR-ABL and the $CD7^{+}CD4^{-}CD8^{-}$ subgroup. P-gp expression was not found to be an indicator of treatment failure in childhood ALL, but was a prognostic signifier in adult ALL. This apparent difference may to be due to a higher use of non-effluxed corticosteroids in the childhood regimen[202,203].

Another haematological disease where P-gp expression is associated with an adverse subgroup is in multiple myeloma (MM). Here a higher frequency of P-gp over-expression has been observed in relapsed MM compared to that seen in *de novo* patients, especially where there has been prior treatment with the P-gp substrates doxorubicin or vincristine. As a consequence to this, P-gp expression has only limited impact on response in *de novo* MM, but has been recognised as a contributing factor in treatment failure[200,204].

1.7 MDR in Non-malignant Disease

1.7.1 HIV/AIDS

Acquired Immunodeficiency Syndrome (AIDS) is a progressive, debilitating and ultimately fatal disease caused by infection with the human immunodeficiency virus (HIV). There are two main strains of the virus, HIV1 and HIV2 and they mainly differ in their epidemiological characteristics[205]. AIDS is characterized by the progressive deterioration of the immune system which results in multifarious pathologies, especially rare secondary fungal infections and skin tumours more commonly associated with patients who are immunosuppressed[206].

The primary target of HIV has been shown to be T-lymphocytes of the $CD4^+$ (T-helper) lineage and in so doing destroys one of the linchpins coordinating the efficient functioning of the immune system. HIV avoids effective elimination by the body's immune mechanisms by attacking one of the cells responsible for orchestrating an antiviral response[207].

Several components of the combined drug therapies available for the suppression of HIV activity are known avid substrates for P-gp efflux, leading to the possibility of therapeutic impairment in individuals who over-express P-gp. The HIV protease inhibitors included in therapeutic regimens such as HAART (highly active antiretroviral therapy) include amprenavir, indinavir, nelfinavir, ritonavir and saquinavir, and they are all P-gp substrates[208,209]. Classical MDR expression of P-gp on the target T-$CD4^+$ cells may result in sub-efficient intracellular levels of drug accumulation, but also ectopic expression of P-gp could lead to reduced tissue deposition of the drugs and cause the formation of

sanctuary zones (such as in the CNS or testes) for HIV drug evasion and subsequent systemic reseeding[210,211].

1.7.2 Parasitic Worms

P-gp expression in the tissues of certain disease causing parasitic worms confers resistance to anti-helminthic drugs used in the treatment of helminth infestation. It has been demonstrated that co-treatment with MDR reversal agents such as verapamil ameliorates this effect and could help to eliminate the infestation[212,213].

1.8 Other MDR Transporters

1.8.1 Multidrug Resistance-Associated Protein 1

MRP1, ABCC1

MRP1 is a 190 kilodalton organic ion efflux transporter consisting of 1531 amino acids encoded by the MRP1 gene located on chromosome 16 (16p13.1)[38,46,214]. It is widely expressed on many human tissues and cancers and it is located on the plasma membrane and on the endoplasmic reticulum and post-golgi vesicles, suggesting an additional intracompartmental efflux role, removing drugs from their intracellular targets[38,215].

MRP1 has a broad efflux substrate spectrum similar to P-gp including daunorubicin, vincristine, and colchicine, but unlike P-gp it can transport compounds that are conjugated to glutathione. Many drugs, including chlorambucil and melphalan, are detoxified and deactivated by the glutathione reductase pathway[216,217]. MRP1 can also transport drugs modified by glucosylation, sulfation and glucuronylation giving it a direct link to other forms of drug modification based resistance[218].

MRP1 may have a role in MDR in several cancers, especially small and non-small cell lung cancer[219], but is not thought to have any individual significance in AML since it is only expressed in 10% of new cases[38,199]. The MRP1 gene expression has been found to be greater in acute lymphoblastic leukaemia (ALL) patients when compared to normal individuals or AML patients, but the prognostic significance of its expression remains unclear[220-222]. It has been suggested that MRP1 may contribute to MDR in AML when co-expressed with P-gp[223,224]. Efflux can be inhibited *in vitro* by CsA, and probenecid and biricodar[46].

1.8.2 Other MRP Family Transporters

ABCC2 (MRP2, cMOAT), ABCC3 (MRP3), ABCC4 (MRP4, MOATB), ABCC5 (MRP5, MOATC), ABCC6 (MRP6), ABCC10 (MRP7). ABCC11 (MRP8), ABCC12 (MRP9)

MRP2 maps to chromosome 10q24 and is associated with the export of organic anions from the liver into bile. It is expressed by canalicular cells and may be involved in conferring drug resistance if overexpressed, although its physiological significance has not been elucidated. Knockout mice for MRP2 develop similar pathologies to the human genetic disease Dubin-Johnson syndrome[225,226]. MRP3 is similar in expression and function to MRP2, and maps to chromosome 17q21.3. Its significance as a contributor to MDR is undetermined[227,228] (Table 1.3).

MRP4 maps to chromosome 13q32 and has a low level of expression in many tissues and cell types and may play a role in MDR in the transport of nucleoside analogues, such as fludarabine and the antiretroviral PMEA, which may lead to resistance to both anticancer and anti-HIV therapies[229-231]. MRP4 has also been identified as being an exporter of prostaglandins and that this function may be inhibited by the action of non steroidal anti-inflammatory drugs[232]. MRP5 gene maps to chromosome 3q27, and is similar to MRP4 in expression and function[231].

MRP6 is expressed in the liver and kidney and maps to chromosome 16p13.1, and although it is involved in the translocation of glutathione conjugates, it has not been shown to be directly involved in MDR[233,234]. However, MRP6 (and MRP2) mRNA has been detected in blasts from AML patients, particularly in

those where the MRP1 gene had been deleted, suggesting a compensatory MDR function[223]. MRP7 maps to chromosome 6q21.1, and has been reported to act as a lipophilic anion transporter, but as yet its function remains unclear[235-237].

MRP8 and MRP9 both map as a cluster to chromosome 16q21.1, and although a human T-cell leukaemia cell line over-expressing MRP8 shows resistance to certain nucleoside drugs, its function is largely unknown[238,239]. MRP9 has two transcripts, one leading to a truncated protein and has been observed being expressed in breast cancer[240]

Table 1.3 Summary of MRP Family ABC Transporters

MRP	Alias	Chromosome	Expressed	Substrates Include	Role in MDR?
MRP1	**ABCC1**	16p13.1	Ubiquitous	Glutatione conjugates	Yes
MRP2	**ABCC2**	10q24	Canalicular cells	Organic anions	Yes
MRP3	**ABCC3**	17q21.3	Liver	Organic anions	Yes
MRP4	**ABCC4**	13q32	Ubiquitous	Nucleotides & nucleoside analogs	?Yes
MRP5	**ABCC5**	3q27	Ubiquitous	Nucleotides & nucleoside analogs	?Yes
MRP6	**ABCC6**	16p13.1	Liver/Kidney	Glutathione conjugates	?No
MRP7	**ABCC10**	6q21.1	Ubiquitous	Lipophilic anion	?
MRP8	**ABCC11**	16q21.1	Most Tissues	?Nucleotide analogs	?No
MRP9	**ABCC12**	16q21.1	Testes/Prostate/Ovaries	?	?No

1.8.3 Breast Cancer Resistance Protein

BCRP, ABCG2, MRX

BCRP is a half-transporter and is encoded by the *ABCG2* gene located on chromosome 4 (4q22)[46,241]. It is distinctive from the other MDR ABC proteins in that it has only one TMD of 6 α-helices and one associated NBD, analogous to half a standard P-gp molecule[52]. BCRP was discovered in MDR cell lines that did not over express P-gp or MRP1 and is associated with mitoxantrone resistance in the breast cancer cell line MCF-7[242], and with methotrexate

resistance[243]. BCRP has been found to form a component of the blood brain barrier[244], is expressed in tissues as diverse as the placenta, intestine and liver[245], and is one of the distinguishing markers for the so called side-band population of haemopoietic stem cells[246].

Studies have been conducted to determine the clinical relevance of BCRP expression in malignant disease[245]. BCRP was found to have no clinical significance in childhood Acute Lymphoblastic Leukaemia (ALL)[247], but studies into its relevance in AML have been mixed: some report a strong association with disease[248-250], while others saw only a negligible correlation[191,251].

1.8.4 Lung Resistance Protein

Lung resistance protein (LRP) is a 110 kilodalton protein, which is encoded on chromosome 16 (16p13.1)[252]. Unlike the previously described MDR transporters, it is not an ABC transporter, but is comprised of a major vault protein subunit complexed with a small RNA molecule and at least two other proteins. LRP is thought to be located on the nuclear membrane and confers resistance by redistribution rather than efflux of drug substrates[38,253].

LRP over-expression has been shown to correlate to shorter progression-free and overall survival in patients with advanced testicular germ-cell tumours[254] and in advanced ovarian carcinoma[255]. LRP over-expression has also been observed in acute leukaemias (AML[256] and ALL[257]), but its prognostic significance remains unclear[258].

The normal physiological role of LRP has not been firmly established. It has been found to be expressed in the kidney, adrenal, heart, lung, muscle, thyroid, prostate, bone marrow and testis, showing considerable overlap with P-gp and MRP1, advancing the case for a xenoprotective function[252,259].

1.9 P-gp Modulation

The current strategy for attempting to ameliorate the effects of P-gp expression in malignant disease is to co-administer a non-cytotoxic P-gp inhibiting agent as an adjuvant to a conventional chemotherapy drug regimen. The P-gp inhibitor, through effective disabling (or modulating) of the P-gp efflux function, can then act as a chemosensitizer by enabling increased net drug uptake by the target cells and so increase cytotoxic efficacy.

P-gp can be modulated by a large range of compounds that can function in two basic modes of action: either to block its function by binding to it allosterically, such as vanadate[260], or to competitively inhibit by acting as a high avidity substrate, such as verapamil[261,262]. Numerous *in vitro* studies have investigated the ability of these substances to modulate MDR, mostly using cell lines and fluorescent dye efflux flow cytometry[263]. Some of these compounds, such as cyclosporin A and its non-immunosuppressive analogue PSC833 have been evaluated in clinical trials[264-266]. They are reported to induce undesirable pharmacokinetic interactions at concentrations necessary for P-gp modulation[267,268].

Table 1.4 summarises the main compounds in use for MDR modulation and shows the development from 1st generation modulators, which were primarily in use for other purposes, such as cyclosporin A. This generation of compounds, although effective *in vitro* could not be readily used in the clinical setting because often the concentrations required for *in vivo* P-gp inhibition were too high and caused deleterious side effects. The 2nd generation modulators were developed usually as structural analogues of 1st generation compounds, but displaying a

much reduced toxicity profile. The 3rd generation modulators are novel compounds screened or engineered to be specific P-gp inhibitors, they present the highest efficacy with the least side effects (Table 1.4).

Table 1.4 Progressive development of P-gp modulators

P-gp Inhibitors	
1st Generation	*(compounds with previous clinical use)*
Verapamil Cyclosporine A Quinine	Calcium channel inhibitor Immunomodulator Anti-malarial drug
2nd Generation	*(structural analogues)*
R-isomer of Verapamil PSC-833 Biricodar (VX-710)	Less toxic than Verapamil Non-immunomodulatory analogue of CsA Inhibits P-gp & MRP1
3rd Generation	*(original compounds)*
Z.3HCL GF120918 Tariquidar (XR9576)	Specific for P-gp Inhibits P-gp & BCRP Anthranilic acid derivative
Other	
Progesterone Rosemary Extract Grapefruit and saville orange juice extract	Polymethoxyflavones

For a MDR modulator compound to be effective as a clinical adjuvant to chemotherapy there are certain criteria that ideally should be met. First, it should be specific as an MDR modulator as accessory pharmacokinetic interactions could lead to side effects and contraindications with certain drug combinations. Whether the compound should be specific for only one type of

MDR transporter is a moot point. Zosuquidar trihydrochloride is specific for only P-gp and not other transporters, while Biricodar is capable of inhibiting both P-gp and another class of MDR transporter, MRP1. The more specific single transporter approach has the advantage of possibly having fewer unwanted side effects and is certainly more useful from a research point of view, while the multi-transporter strategy may be clinically more effective in tumours that express more than one MDR effector mechanism (Table 1.5). Secondly, the modulator should have no cytotoxic effect at the concentrations used.

Table 1.5 MDR modulators and their transporter selectivity. Adapted from Dantzig *et al*, 2003

Modulator	P-gp	MRP1	MRP2	BCRP
Z.3HCL	+	-	-	-
PSC-833	+	-	+	-
VPM	+	-/+	-/+	-
CsA	+	-/+	-	-
Biricodar (VX-710)	+	+	-	-
GF120918	+	-	-	+
Tariquidar (XR9576)	+	-	NR	NR
FTC	-	-	-	+
MK-571	-	+	+	-
Probenecid	-	+	+	-

Key: + Inhibition; - no inhibition; -/+ weak inhibition in some circumstances; NR not reported.

1.9.1 CD56$^+$ NK Cells

A valuable tool for evaluating the efficacy of a P-gp modulator in cancer and leukaemia patients is the CD56$^+$ Natural Killer cell because of its inherently high P-gp expression. It is ideally suited for use as part of a flow cytometric fluorescent dye efflux assay to gauge *in vitro* efflux suppression. A Rhodamine 123 (R123) assay utilizing the relative fluorescent intensity of patient derived CD56$^+$ NK cells was used for the pharmacodynamic arm of the clinical trials that formed the core of this research project[124,269,270].

Another means of overcoming the MDR effect could involve altering a drug's structure in some subtle way so that it retains its pharmacological efficacy but exhibits a reduced avidity for P-gp efflux. The addition of a succinate group at the C10 position of paclitaxel produces a new compound that has enhanced penetration across the blood-brain barrier (BBB) due to its reduced interaction with P-gp compared to the original structure[271].

Other techniques, such as the transfection of an anti-MDR1 ribozyme into P-gp expressing cells have also been shown to be able to reverse MDR efflux. The ribozyme is a RNA molecule (oligonucleotide) with enzymic activity that is able to degrade the MDR1 mRNA transcript, thus reducing expression of the P-gp protein[272-274].

1.10 Zosuquidar Trihydrochloride

Zosuquidar trihydrochloride (Z.3HCL) is a potent and selective third generation inhibitor of P-gp. It is a difluorocyclopropyldibenzosuberane quinoline derivative ((R)-4-[(1aR,6R,10bS)-1,2-difluoro-1,1a,6,10b-tetrahydrodibenzo[a,e]-cyclopropa[c]cycloheptan-6-yl]-α-[(5-quinoloyloxy)methyl]-1-piperazineethanol trihydrochloride) and has a molecular formula of $C_{32}H_{31}F_2N_3O_2 \cdot 3HCl$ and a molecular weight of 637.0 (Figure 1.5). It is produced by Eli Lilly & Company with a CAS registry number of 167465-36-3. It was previously known as LY335979 and before that as RS-33295-198 and was first described by Slate *et al* in 1995[275].

Z.3HCL has been described as possessing many characteristics that qualify it as an 'ideal modulator' of P-gp: it binds with high affinity and specificity to P-gp and it is not a substrate for efflux, ensuring that it is effective over a suitably extended timescale[276]. The exact inhibitory mode of Z.3HCL is still unclear, but it is known that it has no significant effect on other multidrug transporters such as MRP1 and BCRP[277]. This specificity, in contrast with many first and second generation modulators, means there are no, or at least much reduced pharmacokinetic interactions with coadministered drugs, thus enabling unaltered dosing regimens[276,278-280].

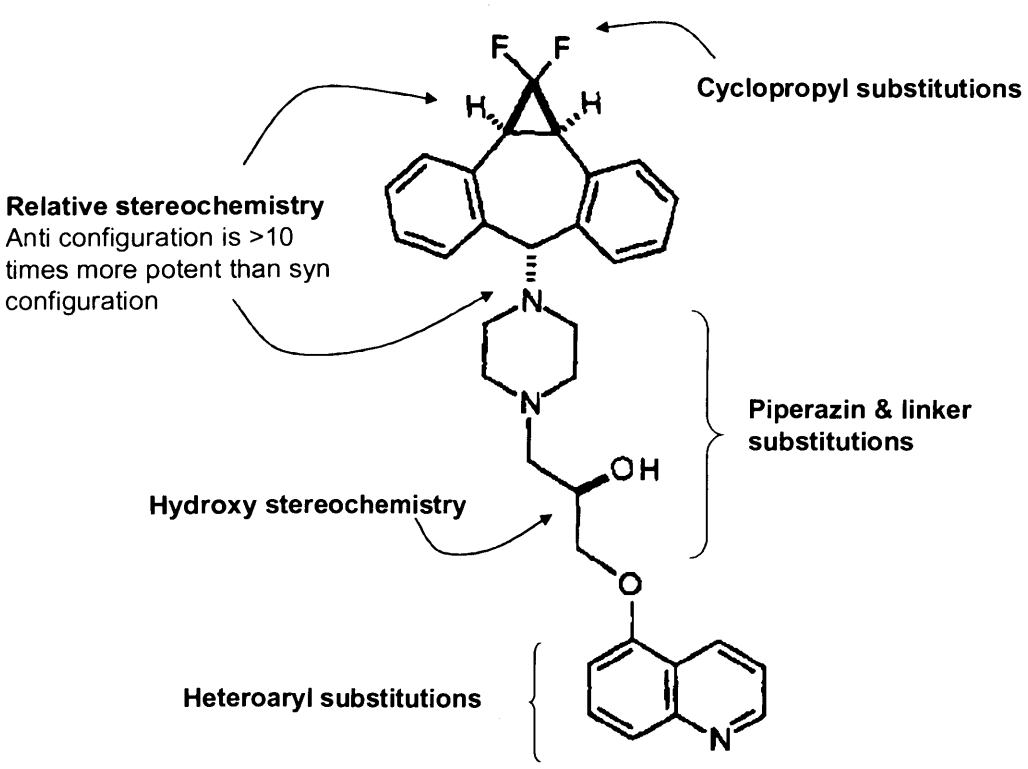


Figure 1.5 Structure-activity diagram of Z.3HCL

Z.3HCL is thought to bind in an allosteric manner to P-gp and in doing so it causes an alteration in the protein's configuration, thus inactivating P-gp efflux function. Z.3HCL binds with a high avidity with concentration of half-maximum inhibition (K_i) of 59nM[276].

In vitro cell line studies and *in vivo* investigations using mouse xenograft models clearly show that Z.3HCL is highly effective at neutralising the protective benefit of P-gp over-expression and enhancing the cytotoxic effects of chemotherapy agents known to be P-gp substrates[281]. For clinical use in human patients, it can be administered either orally or via IV.

1.10.1 Clinical Trials of MDR Modulators

Z.3HCL has been clinically investigated for use as a chemotherapeutic adjuvant for overcoming MDR, but the phase III studies are still ongoing. However, there have been nine randomised clinical trials to date that have included other MDR modulators in conjunction with chemotherapy: 5 on leukaemias (2 AML[267,282], 2 Myeloma[283,284], 1 acute leukaemia (AML, ALL, MDS in transformation[285]), and 4 on solid tumours (2 breast cancer[286,287], 2 lung cancer[288,289]). Out of these, only three trials observed a significant benefit from the incorporation of the MDR modulator compared with the unmodulated arm of the study[264] (Table 1.6).

Of those that did show a benefit, the SWOG AML study (List *et al*, 2001) showed that, although remission rates were not significantly improved (39% v 33%, P=0.14), relapse-free survival (34% v 9% at 2 years, P=0.031) and overall survival (22% v 12%, P=0.046) were significantly increased in the CsA arm[282]. The Solary study with quinine again showed no significant difference in the rates of CR (52.8% v 45.5%, P=0.19) but did observe a modulator benefit for failure of the regimen due to blastic persistence or increase, which was higher in the control group (61/154 patients) than in the quinine group (45 / 161, P=0.04)[285]. They concluded that the increased benefit of modulation by quinine could have been masked by the concomitant increase in toxicity. However, a later study by Solary using quinine in AML did not find any benefit in overall survival, but did observe an increase in CR for patients identified as having P-gp efflux[290] in vitro. The Millward study in lung cancer with VPM showed that the median survival from start of treatment was significantly better in the verapamil arm (P=0.02)[288].

There were also negative results arising from the use of a MDR modulator, in particular the PCS-833 study in patients with AML reported by Baer *et al*, 2002. In this case, the modulator arm of the study was closed prematurely, after the randomisation of 120 patients, due to adverse rates of remission, non-response and death compared with the un-modulated arm (P=0.08). However, further analysis showed that DFS and overall survival did not differ significantly between the two arms and were consistent with historical studies[267].

It is interesting to note that when analysis was restricted to a subset of patients tested for *in vitro* P-gp efflux function, those exhibiting efflux function had a worse outcome than those with no efflux function in the un-modulated arm (P=0.03). However, in the modulated arm there was no difference between the two groups. Furthermore, patients who exhibited P-gp efflux function had a greater median DFS in the modulated arm than in the un-modulated arm (14 months and 5 months, respectively), although this difference was not shown to be statistically significant (P=0.07)[264,267].

The above studies show that although there may have been an overall increase in toxicity associated with the use of some modulators, characterised by deleterious pharmacokinetic interactions, a positive benefit could be observed by restricting the results to outcomes from P-gp expressing patients. It can therefore be argued that subsequent trial design should incorporate a targeted approach to the administration of MDR modulators to only those patients who might gain any benefit: those with primary resistance (P-gp functionally positive by *in vitro* analysis at presentation) with a crossover schedule for those patients who develop secondary resistance during the course of the treatment.

Table 1.6. Randomised clinical trials investigating P-gp reversal in cancer and leukaemia, indicating those that found a significant advantage in favour of the modulator.

Disease	Study	Inhibitor	*n*	Chemo	Response - Inhibitor	Response + Inhibitor	Significant Benefit Seen
AML	List *et al*, *2001*	CsA	226	DNR & ARA-C	33%	39%	RFS: P=0.031 OS: P=0.046
	Baer *et al*, 2002	PSC833	120	ADE	46%	39%	No
Myeloma	Dalton *et al*, 1995	VPM	200	VAD	41%	36%	No
	Sonneveld *et al*, 2001	CsA	75	VAD	49%	53%	No
Breast Cancer	Wishart *et al*, 1994	Quinidine	233	Epirubicin	44%	43%	No
	Belpomme *et al*, 2000	VPM	99	Vindesine & 5-FU	11%	27%	No
Acute Leukaemia	Solary *et al*, 1996	Quinine	315	MIT & ARA-C	46%	53%	BP: P=0.04
NSCLC	Millward *et al*, 1993	VPM	68	Vindesine & Ifosfamide	18%	41%	MS: P=0.02
SCLC	Milroy *et al*, 1993	VPM	220	CAVE	80%	83%	No

Abbreviations: ADE= ARA-C, DNR, Etoposide; CAVE= Cyclophosphamide, DXR, VNC, Etoposide; OS= overall survival; RFS= relapse free survival; BP= blastic persistance; MS= median survival.

1.11 Malignant Diseases Involved in Study

1.11.1 Acute Myelogenous Leukaemia – AML

AML is a group of malignant proliferative diseases affecting the myeloid lineage of leukocytes. It is characterized by an accumulation of immature haemopoietic cells in the bone marrow and blood, and the malignant infiltration of tissues and organs, particularly the liver and spleen. Although patients often present with a normal or elevated leukocyte count, the underlying pathophysiology results in the decreased production of normal blood cells leading to varying degrees of anaemia, thrombocytopenia, neutropenia, [291].

AML can be categorized along morphological criteria into 8 main subsets, using the FAB (French, American, British) classification: M0 – M7. The predominant focus of this study was with regard to P-gp expression in AML, a disease in which MDR is a recognised clinical problem[199,292]. A general overview of the precursor cell lineages leading to the cells affected by the haematological malignancies under discussion are shown in Figure 1.6. This diagram is purely schematic and does not take into account that in AML the leukaemic event is thought to occur in multipotent progenitors largely independent of the observed FAB type[293,294].

1.11.2 Chronic Lymphocytic Leukaemia – CLL

CLL comprises a group of several malignant disorders affecting leukocytes of both lymphoid lineages, as both T and B cells can be involved, although the latter is predominant. It is characterised by the appearance of mature looking blast cells circulating in the peripheral blood and there is considerable overlap

with the non-Hodgkin's lymphomas, depending upon the relative tissue distribution[295,296].

B-CLL, the most common leukaemia in the Western Hemisphere, is characterized by the accumulation of long-lived, relatively mature monoclonal B cells, which express CD5, CD19, CD23 and weak surface IgM (or IgD) immunoglobulin[297].

Relatively few drugs used in the treatment of B-CLL are efflux substrates for P-gp and so this mechanism of MDR is not thought to play a major role in the development of drug resistance in CLL.

1.11.3 Multiple Myeloma – MM

Multiple Myeloma is a neoplastic clonal expansion of bone marrow plasma cells which is pathologically characterized by lytic bone lesions, accretion of plasma cells in the bone marrow, humoral immunodeficiency, anaemia, and the presence of monoclonal protein in the serum and urine[298,299].

Plasma cells are antibody producing cells derived from terminally differentiated B-lymphocytes. Antigenic stimulation causes a small subset of reactive naïve B-lymphocytes, or reactivated B-memory cells, to expand clonally, migrate from the lymph nodes to the bone marrow and differentiate into plasma cells. Typically they express CD38 and CD138 cell surface markers.

Although the disease remains largely incurable, medical intervention has

increased the median survival of patients from just seven months to five years. Contemporary treatment regimens include vincristine, doxorubicin, cyclophosphamide and a high dose corticosteroid, either dexamethasone or methylprednisolone[300]. Many of the drugs used for the treatment of MM are P-gp efflux substrates, and therefore may benefit from the adaptive use of P-gp modulators. Novel agents such as thalidomide and bortezomib are not thought to be affected by P-gp expression. Indeed the proteasome inhibitor bortezomib has been shown to modulate P-gp expression, possibly by interfering with NF-κB signalling pathways[301,302].

1.11.4 Non-haematological Tumours

There are numerous solid tumour cancers that are affected by MDR, especially those cancers that are derived from tissues that naturally express P-gp in significant quantities: cancers of the gastrointestinal (GI tract), including liver and pancreatic cancer; cancers of the genitourinary system, including cancers effecting the kidney, ovary and testicle[215].

Patients enrolled on the two Z.3HCL solid tumour clinical trials which formed a part of this research project (JTAC and JTAM) had either small cell lung cancer, non-small cell lung cancer or breast cancer[279,303].

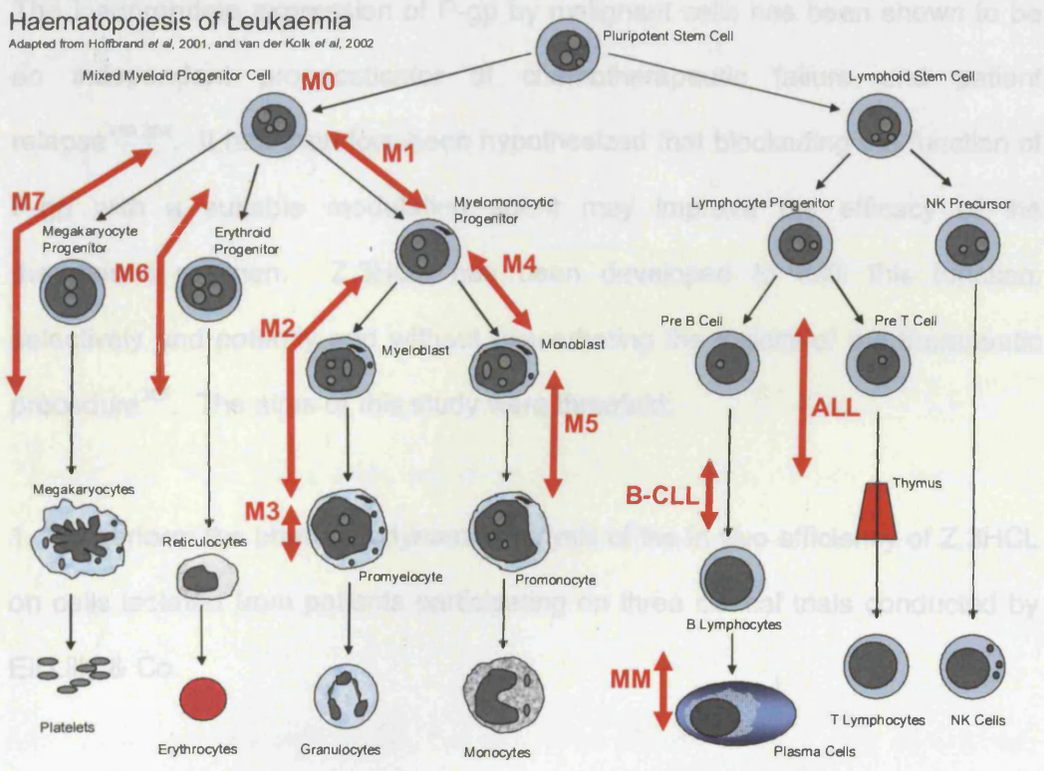


Figure 1.6 Cell lineage haematopoiesis

1.12 Aims

The inappropriate expression of P-gp by malignant cells has been shown to be an independent prognosticator of chemotherapeutic failure and patient relapse[199,304]. It has therefore been hypothesized that blockading the function of P-gp with a suitable modulating agent may improve the efficacy of the therapeutic regimen. Z.3HCL has been developed to fulfil this function, selectively and potently and without exacerbating the toxicity of the therapeutic procedure[305]. The aims of this study were threefold:

1. To perform the pharmacodynamic analysis of the in vivo efficiency of Z.3HCL on cells isolated from patients participating on three clinical trials conducted by Eli Lilly & Co.

2. To characterize Z.3HCL as an inhibitor of P-gp functional efflux in cells isolated from patients with a variety of haematological malignancies. It was envisioned that the data generated may indicate further avenues of clinical investigation.

3. To investigate the putative role played by P-gp in the inhibition of caspase-dependent apoptosis, and to discover whether Z.3HCL is effective at modulating this effect.

2. Materials and Methods

2.1 Clinical Trial Assays

2.1.1 Rhodamine 123 Efflux Assay

Peripheral blood samples were taken from the patients using one or, when possible, two 9ml Becton Dickinson Cell Preparation Tubes (CPT), which were then processed according to the manufacturer's recommendations. These were taken, depending upon the Z.3HCL dosing group at: 0, 1, 6, 8, 24 or 0, 0.5, 3, 6, 12 and 24h. The tubes were centrifuged at 1500g (~2900 RPM in a bench-top Centaur centrifuge) for 20 minutes at ambient temperature to allow density gel separation of the mononuclear cell fraction.

After three washes with HBSS, the cells were aliquoted into 2 sets of 3 tubes. One set of tubes was spiked with 10μl (100nM final) Z.3HCL, which would ensure maximal *in vitro* P-gp inhibition. The 'buffer' set received 10μl RPMI as a control. The cells were then incubated with 50ngml^{-1} R123 (Molecular Probes, R-032) for 90min at 37°C, washed with ice cold wash buffer (PBS + 0.1% BSA) and incubated for 15min at 37°C with anti-CD56 Cy-chrome antibody (Pharmingen, 31668X). After red cell lysis, (1ml ddH_2O for 30 seconds followed by 1ml x2 PBS), fluorescence was read on a Becton Dickinson FACScan using CellQuest software, and the data analysed and compensated with Verity Winlist 4.0 software (Figure 2.1).

The *in vivo* efficacy of Z.3HCL was measured using CD56$^+$ Natural Killer (NK) cells as surrogate reporter cells because of their intrinsically high P-gp expression. This was particularly necessary for the solid tumour trials because,

due to the nature of the disease, direct sampling of malignant material was not practical.

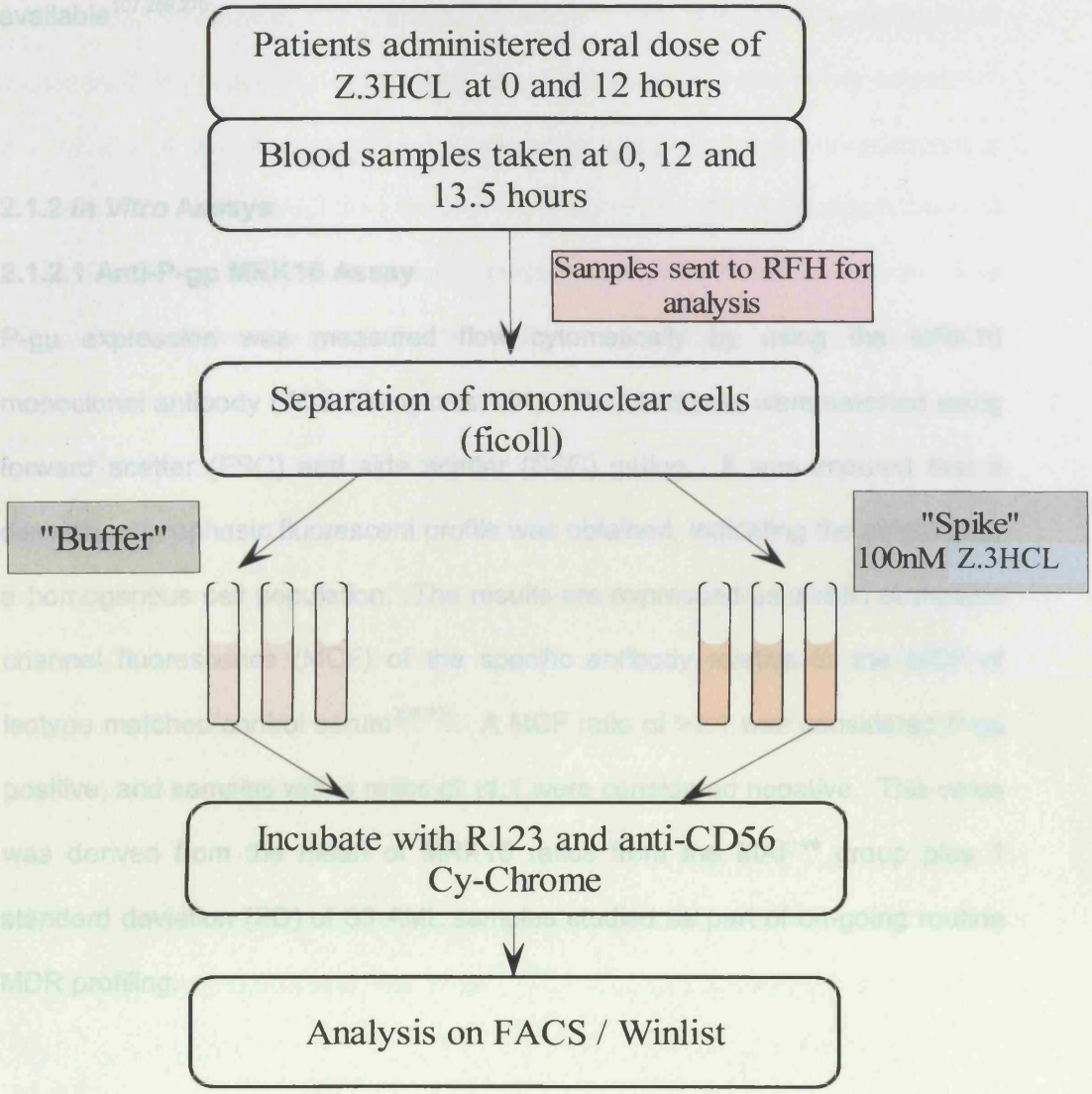


Figure 2.1 Protocol for mononuclear cell preparation for flow cytometry Assay

R123 is a P-gp substrate and the relative brightness of a cell read in a flow cytometer is a direct indication of the cell's accumulation function. For the AML

study, anti-CD33 labelling was used in parallel to anti-CD56 in the R123 assay for direct gating of blast cells where sufficient patient material was available[107,269,270].

2.1.2 *In Vitro* Assays

2.1.2.1 Anti-P-gp MRK16 Assay

P-gp expression was measured flow cytometically by using the MRK16 monoclonal antibody (TCS Biologicals, UK). The blast cells were selected using forward scatter (FSC) and side scatter (SSC) gating. It was ensured that a discrete, monophasic fluorescent profile was obtained, indicating the selection of a homogenous cell population. The results are expressed as a ratio of median channel fluorescence (MCF) of the specific antibody relative to the MCF of isotype matched control serum[306,307]. A MCF ratio of >1.1 was considered P-gp positive, and samples with a ratios of ≤1.1 were considered negative. This value was derived from the mean of MRK16 ratios from the MAF^{-ve} group plus 1 standard deviation (SD) of 53 AML samples studied as part of on-going routine MDR profiling.

2.1.2.2 Calcein-AM Functional Assay

A functional P-gp accumulation assay using the fluorescent dye Calcein-AM (Molecular Probes) a acetoxymethyl ester of calcein, co-incubated with either Z.3HCL or Verapamil (VPM) was used to assess *in vitro* P-gp activity. The calcein-AM accumulation assay has been widely used to detect P-gp functional activity in AML cells[308,309]. Both the Z.3HCL and the VPM stock solutions were

stored in DMSO and multiple dilutions in RPMI were used to obtain the final working solutions to avoid any cytotoxic effects of DMSO. Once inside cells, calcein-AM is cleaved by cellular esterases and the calcein derivate is fluorescent. P-gp actively effluxes calcein-AM but not the derivative calcein[310]. The MCF's of the modulated and unmodulated cells was used to generate a Multidrug Activity Factor (MAF), a ratiometric figure (0 – 100, with significance at ≥ 10.0) denoting the ability of each modulator to correct calcein efflux. The higher the MAF, the more effective the inhibitor or the greater amount of P-gp.

$$\mathbf{MAF = ((MCF_m - MCF_c) / MCF_m) \times 100}$$

Where MCF_m is the median channel fluorescence of the modulated cells, and MCFc is the median channel fluorescence of the unmodulated control cells. Samples with a MAF of ≥ 10 were considered to be P-gp positive, and samples with a MAF <10 were considered P-gp negative.

MRP1 is able to efflux both calcein-AM and the fluorescent calcein derivative, but at a markedly slower rate than P-gp[311,312].

Calcein-AM was obtained from Molecular Probes and stored at -20°C at a stock concentration of 0.1mM in DMSO (Sigma) as 10μl aliquots. Immediately prior to use, the 10μl was diluted with 190μl sterile saline to obtain a working solution (w/s) of 5μM. This was added to a falcon FACS tube of 5 x 10^5 cells in 1ml RPMI to giving a final concentration of 50nM.

2.1.2.3 MTT Drug Sensitivity Assay

The ability of viable cells to reduce 3,4,5-dimethylthiazol-2,5,-diphenyl tetrazolium bromide (MTT, Sigma, UK) was used as a measure of drug sensitivity. The amount of purple formazan produced by the process is linearly proportional to the number of viable cells when read on a suitable plate reader (Anthos Labtec Instruments, Austria)[313].

Drug sensitivity profiling against ARA-C, DNR and mitoxantrone (MIT) for AML cells by MTT assay was conducted whenever sufficient patient material was available. These were performed in 96 well microtitre plates with the patient's cells incubated with 6 concentrations in serial dilution of cytotoxic drugs, with and without 100nM Z.3HCL to generate comparative IC_{50} levels. At 72h 10μl of MTT (5mg/ml) was added for an additional 4 hours. The precipitated purple MTT formazan was centrifuged for 5 minutes at 600x*g*. The supernatant was removed and the formazan pellet was dissolved in 100μl DMSO (dimethyl sulfoxide). The statistical software package Prism 3 (Graphpad) was used to perform a one-tailed Wilcoxon Signed Rank Test between modulated and unmodulated IC_{50} curves to determine if any differences in drug sensitivity are statistically significant[143].

CLL cells were tested against CHL, DNR and FLU. Neither CHL nor FLU are P-gp substrates. MM cells were tested against DNR, MELP, and PRED or VNC.

The use of the MTT assay for the apoptosis studies detailed in chapter 5 differed from the MDR MTT assay in that no drug dilutions were used and no IC_{50} was calculated. Cells, typically following γ-radiation exposure, were assayed with the

MTT in order to elucidate relative cell viability in comparison with a control population of cells.

2.2 *In Vitro* Study Procedures

2.2.1 Mononuclear Cell Preparation Using Lymphoprep™

Peripheral blood or bone marrow aspirate was taken by clinical staff, preferentially in 30ml universal tubes containing 200 units of preservative free heparin; although 9ml lithium heparin 'vacutainer' tubes were also employed, depending upon availability. Mononuclear cells were separated by density gradient centrifugation on Lymphoprep™ (*Nygaard, Norway*). Employing sterile conditions in a class II flow cabinet, the peripheral blood was diluted with an equal volume of HBSS. A 15ml, 30ml or 50ml tube was employed depending upon the sample volume, into which was poured an amount of Lymphoprep equal to the sample volume. The sample blood was then carefully pipetted down the inside wall of the tube so that it formed a layer on top of the Lymphoprep and maintaining a clearly defined interface. The sample was then spun in a bench-top centrifuge (*MSE Centaur*) at x600g (2500 rpm) for 20 minutes at room temperature.

The mononuclear cell population (i.e. lymphocytes, monocytes and blast cells) was then carefully removed from the interphase layer to a fresh tube using a syringe or pipette ensuring minimal contamination with Lymphoprep. The cells were then in resuspended HBSS and spun at x480g (2000 rpm). The cell pellet was then inspected for red cell contamination, in which case a Red Cell Lysing solution was employed, followed by a wash in HBSS. If no significant RBC contamination was present, the cells were washed twice in HBSS and then resuspended in RPMI + 10% FCS, at a volume appropriate to the cell pellet size (typically 10ml – 20ml). The cell solution was kept for up to 4 hours at 4°C to minimize cell deterioration until use.

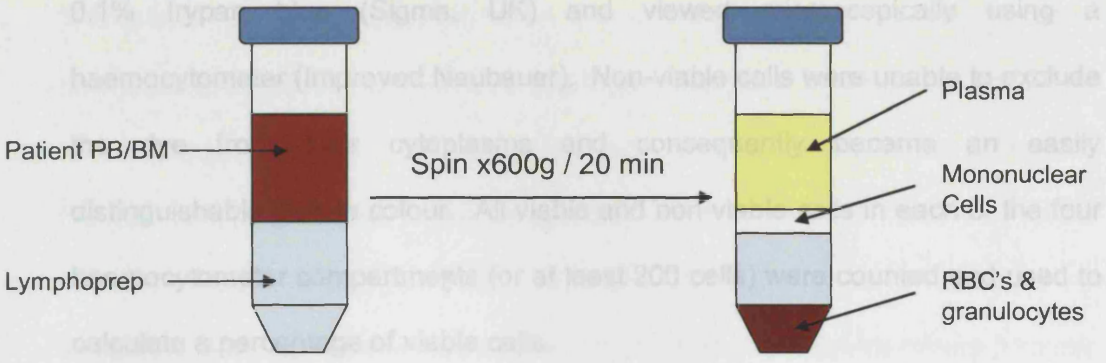


Figure 2.2 Mononuclear cell preparation from Lymphoprep

2.2.2 Other MDR Transporter Measurement

MDR Transporter expression was studied on a FACScan (Becton Dickinson) using the following monoclonal antibodies:

MRP1 - MRP6 antibody, IgG_1 Isotype

LRP - LRP56 antibody, $IgG2_b$ Isotype

BCRP - BXP21 antibody, IgG_{2a} Isotype

The results were expressed as a ratio of MCF of the specific antibody relative to the MCF of isotype matched control serum.

2.2.3 Trypan Blue Cell Viability Assay

The viability status of cells was analysed using a trypan blue dye exclusion assay. 10µl of the $1x10^6ml^{-1}$ cell suspension was mixed with an equal volume of

0.1% trypan blue (Sigma, UK) and viewed microscopically using a haemocytometer (Improved Neubauer). Non-viable cells were unable to exclude the dye from their cytoplasms and consequently became an easily distinguishable blue in colour. All viable and non-viable cells in each of the four haemocytometer compartments (or at least 200 cells) were counted and used to calculate a percentage of viable cells.

2.2.4 JC-1 Assay

JC-1 is a biphasic fluorochrome used to determine mitochondrial membrane potential (Ψ_m) as an indicator of apoptotic modulation by P-gp and the inhibition of that effect by Z.3HCL. It emits at two concentration dependent spectra, corresponding to a green signal (cytoplasm - FL1) and a red signal (mitochondria – FL2). A ratio of the red signal to the green signal (from FL1 / FL2 quadrant percentages) gives a measurement of the relative strength of the Ψ_m.

At low relative concentrations, JC-1 exists as a monomer (green fluorescent signal), but beyond a certain concentration threshold, the monomers begin to form aggregated stacks called J-aggregates (red fluorescent signal). Since J-aggregate formation increases linearly with applied membrane potential over the range of 30–180mV, this phenomenon can be exploited for potentiometric measurement of apoptotic mitochondrial depolarization[314-316].

One distinctive feature of the early stages of apoptosis is the disruption of active mitochondria (via BAX, BID etc). This mitochondrial disruption includes changes in $\Delta\Psi$m and alterations to the oxidation–reduction potential of the mitochondria.

Changes in $\Delta\Psi m$ are presumed to be due to the opening of the mitochondrial permeability transition pore allowing passage of ions and small molecules. The resulting equilibration of ions leads to the decoupling of the respiratory chain and the release of cytochrome C into the cytosol.

In healthy cells, JC-1 enters the negatively charged mitochondria where it forms concentration dependent liquid crystal aggregates that emit a red fluorescence. In cells undergoing apoptosis, where the mitochondrial potential has collapsed, JC-1 exists monomerically throughout the cell, and when dispersed in this manner JC-1 fluoresces green. Using flow cytometry, changes in $\Psi\Delta m$ are indicated by measuring the red / green intensity ratio.

JC-1 (Molecular Probes, UK) was stored at a stock solution of 1mM in DMSO.
A working solution (w/s) of 50 μM was prepared fresh from 10μl stock + 190μl saline. 10μl of w/s was used in 1ml cells (0.5 x 10^6 cells) making a final concentration of 0.5μM. This was then incubated at 37°C / 15min in dark (water bath). The cells were then washed twice in ice cold PBS, and kept on ice away from direct light until analysed on FACScan with 2-colour online compensation.

The flow cytometry data was reported as the ratio of percentage red signal against the percentage green signal as ascertained from a quartered dot plot displaying FL1 (green) against FL2 (red) along each axis.

2.2.5 Western Blots

The caspases, and in particular caspase 3, can be considered to be the effector molecules of the apoptotic process. They exist as inert or low activity precursors within the cell. On activation, a cascade of proteolytic action leads to the cleavage of numerous intracellular components essential for normal function. This renders the cell a condensed and fragmented apoptotic body readily absorbed by surrounding cells or macrophages.

One of the first recognised and most commonly cleaved substrate is the enzyme poly-ADP-ribose-polymerase (PARP, 116kDa) whose proteolytic cleavage into 24kDa and 85kDa fragments early during the apoptotic process is one of the first detectable events. PARP plays a role in DNA repair and replication, cell proliferation, and differentiation, and its cleavage has a deleterious effect on its function. PARP is known to be cleaved by the apoptotic effector caspase-3.

PARP cleavage, considered to be a hallmark of apoptosis, was measured using western blotting by taking a ratio of the apoptosis specific p85 fragment against the uncleaved PAPR p116 expression. Caspase 3 activation, another hallmark of apoptosis, was also measured initially in the study, and normalised against β-actin.

Protein lysate was obtained from prepared cells using 1ml whole cell lysing solution (WCL) (20mM Hepes-KOH [pH 7.5], 50mM NaCl, 2% nonidet P40, 0.5% sodium deoxycholate, 0.2% sodium dodecyl sulfate (SDS), 1mM sodium vanadate, 10mM sodium fluoride, 1mM ethylene glycol tetra-acetic acid (EGTA)) with 2μl DTT and 10μl PMSF. It was added at 80μl WCL per $10x10^6$ washed

cells, incubated on ice for 15 min and then spun in a benchtop refrigerated microfuge at 4°C for 15 min at 14000 rpm. The supernatant was removed and the lysate stored at -80°C until use.

A protein standard curve was performed in order to determine 20μg protein loading from cell lysate for loading onto SDS PAGE gel lane. Protein concentration was quantified using the Bio-Rad protein assay kit (Bio-Rad, UK), which utilized the proportional colour change of a dye in response to the concentrations of protein and measured using a spectrophotometer at 595nm. Bovine serum albumin (BSA) (Sigma, UK) was used as a protein standard for the making of a standard curve.

Tris Glycine 4-12% precast gradient gel plates were assembled according to the manufacturer's instructions (Invitrogen). The protein concentration was measured, and 20μg of protein samples were made up with 1 x SDS sample buffer (Invitrogen), 80mM DTT reducing agent and ddH_2O. Samples were heat denatured for 10 minutes at 80°C in boiling water bath, centrifuged briefly and loaded into gels using a Hamilton syringe. NuPAGE (Polyacrylamide gel electrophoresis) Gels (Invitrogen) were run at 200V using 1x MOPS (N-morpholino propanesulfonic acid) running buffer (Invitrogen) and NuPAGE running buffer antioxidant (Invitrogen) for approximately 45mins.

Two Whatman filter papers and one nylon membrane (Hybond C, Amersham) per gel, cut in the same dimension as the gel were soaked individually in blotting buffer (Bicine 25mM, Bis Tris 35mM, Ethylene diamine tetra-acetic acid (EDTA) 1mM, 10-20% methanol, pH7.2 and 200μl of antioxidant per 200ml of transfer

buffer). The gel was placed on top of one filter, followed by nylon membrane then another filter paper, with care taken to avoid trapping air bubbles between layers. The gel, membrane and filter papers sandwiched with blotting pads pre-soaked in transfer buffer were placed in a blot module (Invitrogen) with transfer buffer. Transfer of proteins was carried out at 25V for approximately 1½h.

After blotting, the nylon membrane was stained with Ponceau-S stain (Sigma, UK) to ensure equal and satisfactory transfer of proteins. The membrane was carefully agitated at room temperature in polyvinylpyrrolidone (PVP) blocking buffer (0.02M Tris, 0.14M NaCl, 2% new-born calf serum and 5% PVP (Sigma) [pH 7.6]) for 1 hour. After blocking, the membrane was placed on top of a plastic sheath, rolled up and placed in a 50ml Falcon tube with 10ml fresh blocking buffer containing primary antibody. The tube was placed in a rotisserie overnight (16-18h) at room temperature.

The blots were probed with rabbit anti-p85, (goat anti-rabbit 2^{o} HRP antibody) and mouse anti-p116 (goat anti-mouse 2^{o} HRP antibody). β-actin and caspase 3 were also probed for in the initial experiments.

After primary antibody incubation, the blot was washed in 1x washing buffer (0.02M Tris, 0.14M NaCl and 0.02% Tween 20) for two 5 min washes followed by one 1hour wash and finally two 5 min washes. The membrane was incubated with horseradish peroxidase linked secondary antibody (DAKO Denmark, 1/5000 dilution) in blocking buffer for at least 1 hour at room temperature. The membrane was finally washed thoroughly in washing buffer for two 5 min washes, a 1hour wash and two final 5 min washes to reduce background.

The ECL (enhanced chemiluminescence) method (Amersham) was used to detect immunoreactive protein bands: the two solutions were mixed in equal proportions (0.5ml each per blot) and pipetted onto the blot, covered, and towelled off after 1min. The blots were then exposed onto x-ray film using a Kodak developer and these films were scanned using a Bio-Rad densitometer using the Quantity One software package, which also calculated the band intensity.

For further antibody staining the blots were stripped by incubation in stripping buffer (162mM Tris [pH 6.7], 2% SDS, 100mM β-mercaptoethanol) for 30 minutes at 50°C.

2.2.6 Annexin-V Assay

The Annexin-V assay kit was used (Caltag MedSystems) to quantitate early apoptotic events flow-cytometrically. This assay uses a FITC conjugated monoclonal antibody specific to externalized membrane phosphatidylserine, which is externalized to the outer membrane surface following apoptotic initiation and is a readily discernable hallmark of early apoptotic changes. The Annexin-V was used in combination with propidium iodide (PI), and the results measured flow-cytometrically on a Becton Dickinson FACScan. Cells, both cell lines and patient PBMC's were treated with varying levels of γ-radiation and incubated for up to 48 hours with and without Z.3HCL. For some experiments utilizing B-CLL cells, the drug CHL was used as the apoptotic initiator. The assay was then

used to look for differential apoptotic sensitivities between control and Z.3HCL modulated samples.

The protocol was adapted from the manufacturer's standard protocol. 0.5 x 10^6 cells per tube were prepared, spun (x1500 rpm / 3mins) and pelleted. The cells were then washed with x1 binding buffer (from kit stock, diluted 1/5 with ddH_2O), spun (x1500 rpm / 3mins) and resuspended in 195μl of x1 binding buffer. Then, 5μl of Annexin-V FITC (from kit) was added to the cells, and they were incubated in the dark at room temperature for 15mins. The cells were then washed in 0.5ml x1 binding buffer, spun (x1500 rpm / 3min) and resuspended in 190μl of x1 binding buffer. After this, 10μl propidium iodide (from kit) was added and the solution mixed. The cells were kept on ice away from direct light until analysed on the flow cytometer using preset FSC, SSC & compensation settings, adjusting as necessary.

The proportion of apoptotic cells was determined by gating on the main cell population and then dot-plot sub-gating with annexin V (FL1) intensity along one axis and PI (FL2) intensity along the other axis. This plot was then quartiled and the percentage of annexin V+ / PI- cells taken. When the flow cytometry data was acquired for all the samples, the data was transferred to a Microsoft Excel spreadsheet file for basic data manipulation and normalisation before being visualised graphically using Prism 4.03, which was also used for the basic statistics and one-tailed paired t-tests. For the CEM/CEMv/VLB cell lines, each experiment contained three replicates for each point and each experiment was repeated on three separate occasions.

2.2.7 Purification of Plasma Cells

Plasma cells (PC) were purified by use of a MACS magnetic bead column system. Paramagnetic microbeads coated with anti-human CD138 (Miltenyi Biotec, UK) were incubated with PBMC's isolated from MM patients (10μl beads per $5x10^6$ total cells, pelleted) and incubated at 4°C for 30 mins. The cells were then washed and resuspended in 3ml cold media and passed through a proprietary magnetic column which retains bead bound plasma cells and allows unbound non-CD138 cells to pass through. The bound plasma cells were then recovered from the column by removing it from the magnetic source and flushing through with ice cold media. A small aliquot of the purified cells was taken and a cell count was determined in a counting chamber (Improved Neubauer Haemocytometer). A morphological test of PC purity was performed from cytospins ($1x10^4$ cells) stained with May-Grunwald-Giemsa (MGG)

2.2.8 Cell Morphology Analysis

Cytospin preparations of no more than $1x10^4$ cells were prepared on glass microscope slides at x400rpm for 2mins on a Shandon Cytospin 2 centrifuge and air dried for a minimum of 10 minutes, but preferably for 1 hour. These were then stained with MGG and viewed microscopically. Apoptotic cells could be distinguished from unaffected cells by morphological changes characteristic of apoptosis such as membrane blebbing and apoptotic bodies. This was used for apoptosis scoring and also for plasma cell purity analysis following MACS magnetic purification.

2.2.9 Cell Line Studies

The human T-cell lymphocytic leukaemia non-MDR cell line CCRF-CEM (CEM) and its P-gp over expressing sub-line CEM/VLB_{100} (VLB) were used for *in vitro* investigation of the efficacy of Z.3HCL and for the preliminary calibration of the assays implemented in the monitoring of patient material. An intermediately P-gp expressing cell line, denoted CEMv, was also established by growing CEM cells in the presence of escalating doses of vincristine (VCR), a cytotoxic drug recognised as an inducer of P-gp upregulation and over expression. A similar strategy was employed in an attempt to produce a P-gp expressing variant of the human acute myeloid leukaemia cell line HL-60 which, despite several attempts was not sucessful.

2.2.10 Statistical Methods

Basic statistic inferences were performed using Microsoft Excel for Windows, which was also used to create the tables. Graphpad Prism 3.03 and 4.03 software for Windows was used to calculate Student's t-tests, Wilcoxon signed rank and Mann-Whitney non-parametric tests for the MTT assays and apoptosis assays, linear regression and correlations for the P-gp and Calcein-AM data and for creating all of the graphs shown. All data sets were tested for normality using the inbuilt function in Prism. Verity Winlist 4.0 for Windows was used for the analysis and off-line compensation of the R123 flow cytometry data. CellQuest software by Becton-Dickenson for Macintosh OS9 was used for the acquisition of all flow cytometry data.

3. Results - Clinical Trials

3.1 AML Trial

3.1.1 Introduction & Study Design

3.1.1.1 Introduction

Several clinical trials have been previously undertaken to investigate the efficacy of MDR modulation, both in solid tumours and in haematological malignancies. These trials studied first generation modulators, in particular cyclosporine A (CsA), and its second generation analogue, PSC-833. Most, if not all of these studies concluded that P-gp modulation was of little or no benefit. However, one study evaluating CsA in elderly patients with AML showed some benefit from modulating P-gp[282], and another evaluating PSC-833 in AML in a phase III trial suggested some benefit, but only in those patients exhibiting P-gp efflux. However, the inhibitor arm of this trial was curtailed prematurely because of increased early mortality associated with PSC-833 combination therapy[267].

The focus of this chapter will concern a phase I trial of Z.3HCL in patients with AML, which was conducted at the Royal Free Hospital. The blood samples for pharmacodynamic (PD) analysis were delivered directly to the lab as soon as they were acquired. The pharmacokinetic (PK) samples were frozen and sent off-site for analysis. Pre-trial samples were also taken from patients at enrolment prior to chemotherapy induction for the purpose of MDR characterisation and drug sensitivity assays. The well-established MDR inhibitor Verapamil (VPM) was used *in vitro* as a standard against which the efficiency of Z.3HCL was contrasted.

The trial was designated JTAN – (B3T-MC-JTAN) by Eli Lilly and was entitled: A Phase 1 Study of Intravenous Z.3HCL in Combination with Daunorubicin and Cytarabine in Patients with Acute Myelogenous Leukaemia or Myelodysplastic Syndrome.

3.1.1.2 Primary Objective of JTAN Trial

To determine a dose of Z.3HCL that can be administered safely, with acceptable toxicity, as a short intravenous infusion (6 hours or less) in combination with DNR and ARA-C in patients with AML or MDS.

3.1.1.3 Secondary Objectives of JTAN Trial

To evaluate the toxicity of Z.3HCL administered in combination with DNR and ARA-C and to determine the pharmacokinetics of Z.3HCL (and its metabolites) and DNR and its metabolite daunorubinicinol when given together and when DNR is given alone. Also to determine any relationship between plasma levels of Z.3HCL and P-gp inhibition as measured *in vitro*.

The study was approved by the Royal Free Hospital Ethics Committee and all patients entered gave written informed consent.

3.1.1.4 Inclusion Criteria

De novo and relapsed patients aged between 18 and 80 years age with morphologically confirmed AML or MDS were eligible for inclusion. All patients had a performance rating of 0–2 on the ECOG (Eastern Co-operative Oncology Group) scale, unless their performance was judged to be a direct consequence of AML/MDS. Any prior chemotherapy must have been completed at least three weeks before enrolment, except for hydroxyurea, in which case the time elapsed should have been six weeks.

Other inclusion criteria included: a negative pregnancy test if female and appropriate contraceptive measures for both females and males to minimise childbearing potential, adequate organ function (Hepatic-bilirubin <1.5 times upper limit of normal (ULN), aspartate transaminase <2.5 times ULN, Renal-serum creatinine <1.5mg/L or greater than calculated creatinine clearance >40ml/min (calculated by Cockcroft and Gault Formulae), multiple-gated acquisition (MUGA) scan or echocardiogram with ejection fraction of ≥45%.

3.1.1.5 Exclusion Criteria

Newly diagnosed APL (AML FAB M3) were not considered for enrolment because of the associated coagulation complications of chemotoxic therapy and the preferred use of ATRA therapy[13]. Patients were not to have had the use of any investigational agent within four weeks prior to enrolment or any serious systemic disorder incompatible with the study, or to have shown any previous cytarabine related neurotoxicity. Other exclusion criteria included pregnancy or

Table 3.1.1 Demographics and Z.3HCL dosing schedule of patients

	Z.3HCL dose	age	sex	diagnosis	Cytogenetics
1	200mg/m² @6h	44	F	de novo M5a	Complex
2	200mg/m² @6h	30	M	de novo M5	Normal
3	200mg/m² @6h	36	M	de novo M4	Trisomy 22 inv(16)
4	200mg/m² @6h	54	M	relapsed M4	Trisomy 21
5	300mg/m² @6h	53	M	refractory M1	t(14:21)
6	300mg/m² @6h	36	F	de novo M2	Trisomy 8
7	300mg/m² @6h	24	M	de novo M6	Normal
8	400mg @ 3h	54	M	relapsed M2	t(6:9)
9	400mg @ 3h	56	M	de novo M1	Normal
10	400mg @ 3h	47	M	de novo M5a	Normal
11	400mg @ 3h	63	M	relapsed M4	Normal
12	400mg @ 3h	25	M	relapsed M1	Trisomy 4
13	400mg @ 3h	69	M	de novo M1	Failed
14	400mg @ 3h	68	M	de novo M1	Normal
15	400mg @ 3h	52	M	de novo M2	Monosomy 7
16	400mg @ 3h	70	F	de novo M2	Trisomy 11, del(X)

breast feeding, unstable angina, myocardial infarction in previous six months, atrial or ventricular arrhythmia or congestive heart failure (CHF).

3.1.1.2 Patient Recruitment

Sixteen AML patients were entered onto the trial: 13 Male and 3 Female with a median age of 52.5y (range 24 - 70y). Six patients had normal cytogenetics, nine had cytogenetic aberrations including two with an unfavourable pattern and one failed the analysis. The median WBC at the start of treatment was 3.4

$x10^9$/L (range 0.9 - 45.4 $x10^9$/L). The patient demographics and inclusion criteria are shown in Table 3.1.1 and Table 3.1.2 respectively.

3.1.1.3 Treatment schedule

ARA-C was infused via a central line at 200mg.m^{-2} for 7 - 10 days. DNR was infused by slow push IV at 50mg.m^{-2} on days 1, 3 and 5. Z.3HCL was given on days 3 and 5 prior to the DNR, as either a 3 or 6h IV infusion to allow assessment of the effect of Z.3HCL on DNR pharmacokinetics (Figure 3.1.1). The patients were split into 3 dosing groups for Z.3HCL: 4 patients received 200mg.m^{-2} over a 6hr infusion; 3 patients at 300mg.m^{-2} over 6hrs, and 9 patients at 400mg flat dosing given over 3hr. The flat dosing of 400mg was derived from initial pharmacokinetic data gathered from earlier phase I solid tumour studies[303].

Patients were monitored for response and adverse events continuously during their in-patient stay for 27 to 74 days and thereafter at intervals of once or twice weekly dependent on clinical status. Bone marrow aspirates were performed at 3-4 weeks when evidence of regeneration was obtained. Complete remission (CR, CR1, CR2) during follow-up was defined as a bone marrow aspirate with less than 5% blasts with subsequent normalisation of blood counts prior to the next course of treatment. Partial remission is defined as the presence of a bone marrow which is regenerating normal haematopoietic cells and contains 5-20% leukaemic blast cells. Toxicity and adverse events were assessed daily using the NIH-NCI Common Toxicity Criteria[317]. Patient follow-up was performed independently of the sponsor of the protocol.

PK analyses were performed on blood samples taken before and at various time intervals at the end of Z.3HCL IV push. These samples were centrifuged according to the protocol and the plasma collected, labelled and frozen at -70°C, before being sent off-site for analysis. These data were collated and discussed by Callies *et al*[318].

Table 3.1.2 Inclusion and exclusion criteria for the JTAN AML clinical trial.

Inclusion Criteria	Exclusion Criteria
Morphological diagnosis AML or MDS	Acute promyelocytic leukaemia
Prior chemotherapy completed >4 weeks ago	No chemotherapy in the last 4 weeks No previous study involving modulation of P-gp
Performance status 0-2	Previous cytarabine related neurotoxicity
Adequate organ function	Unstable angina, uncontrolled AF or arrhythmias
Age 18 - 80 years	Serious concomitant disease
Negative pregnancy test and/or adequate contraception where appropriate	Pregnancy or breast-feeding

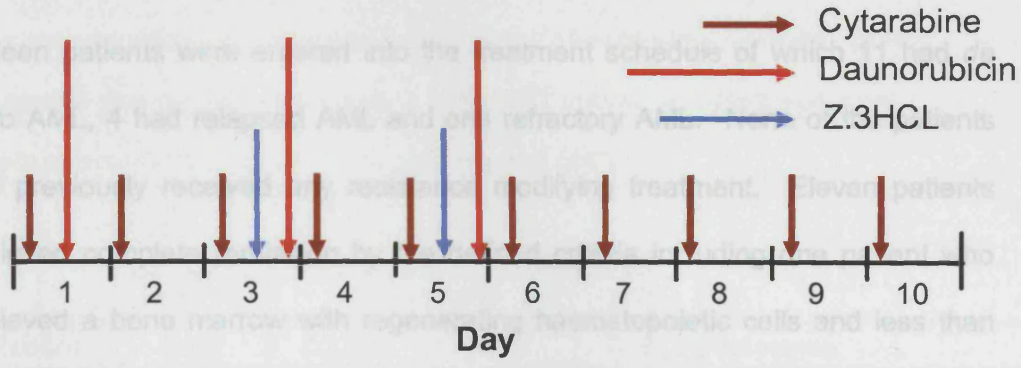


Figure 3.1.1 JTAN Drug and modulator dose schedule. The arrows represent the times when the different drugs were administered. Z.3HCL was given prior to DNR on days 3 and 5, while DNR was given without Z.3HCL on day 1.

3.1.2 Results

3.1.2.1 Clinical Response

Sixteen patients were entered into the treatment schedule of which 11 had *de novo* AML, 4 had relapsed AML and one refractory AML. None of the patients had previously received any resistance modifying treatment. Eleven patients achieved complete remission by the defined criteria including one patient who achieved a bone marrow with regenerating haematopoietic cells and less than 5% blasts but who then died of a probable pulmonary embolus prior to the normalisation of blood counts (ANC $1.7x10^9$/L and platelets $31x10^9$/L). One other patient achieved a partial remission. These data, along with the *in vitro* assay results, are summarised in Table 3.1.3.

The median survival for the 12 patients was 559 (range 38-906) days. Five of the 11 patients later died: 3 of progressive disease post relapse and 2 of infection post mini bone marrow transplant. All 6 patients still alive with a median survival of 722 (range 510-906) days in CR had *de novo* AML. There were 3 deaths prior to assessment of remission status: 2 related to AML (both patients had poor risk cytogenetics) and one, as described above, of probable pulmonary embolus. The median time to neutrophil recovery (>$0.5x10^9$/L) was 21 days and to platelet recovery (>$50x10^9$/L) was 23 days.

3.1.2.2 Toxicity Assessment

In general, Z.3HCL was reported to be well tolerated in combination with DNR and ARA-C. Toxicities and adverse events were graded by NIH-NCI Common Toxicity Criteria and are shown in Table 3.1.4

Table 3.1.3 Results of the *in vitro* studies: P-gp expression (MRK16 assay); accumulation function (calcein-AM assay); and drug sensitivity (MTT assay). Also shown is the corresponding initial response to treatment (CR = complete remission, PD = progressive disease, ND = not done). + in the MTT column denotes a significant sensitisation to DNR observed in the presence of Z.3HCL

	MRK16	Calcein-AM		MTT	
Patient	P-gp	Z.3HCL	VPM	DNR	Response
1	-	+	+	-	CR1 - PD - Died
2	-	+	-	-	CR1
3	+	+	+	-	CR1
4	-	-	-	ND	PD - Died
5	-	ND	ND	ND	Fungal Infection - Died
6	-	-	-	ND	CR1
7	-	-	+	ND	CR1
8	-	-	+	-	CR2
9	+	+	+	+	CR1
10	-	+	-	-	CR1
11	+	+	+	-	PD - Died
12	-	+	+	+	CR1
13	-	-	+	-	PE - Died
14	+	+	+	+	CR1
15	+	+	+	ND	PD - Died
16	+	-	+	+	CR1
% Positive	37.5%	60.0%	73.3%	36.4%	

Four patients experienced Grade 3 and Grade 4 toxicities and there were no on-study deaths. Adverse events not attributed to Z.3HCL were mostly predictable following chemotherapy. All patients experienced neutropenic fever, 7 patients experienced nausea and vomiting, 4 had mucositis, 5 diarrhoea and 6 patients exhibited abdominal pain. Palmar rash was observed in 6 patients (2 both

palmar/plantar) with plantar pain experiences in 2 individuals. One additional patient had palmar pain in the absence of any rash. Three other patients had a macular body rash; one had anorexia and one exhibited peripheral oedema. Significant laboratory abnormality was experienced in 2 patients in the form of hyperbilirubinaemia. Both resolved spontaneously though one was reported as a serious adverse event (SAE). This patient also had a SAE of renal failure; however baseline renal and liver functions had been elevated.

The main toxicity seen with the combination therapy of ARA-C and DNR with Z.3HCL was neurological. Two patients experienced grade 3 hallucinations or confusion during the 24h after infusion of Z.3HCL. These patients consequently received only the first dose of Z.3HCL. The first (patient 8) was a patient with relapsed AML who developed vivid hallucinations, bad dreams and fatigue: the patient had never experienced similar symptoms during his previous 4 courses of chemotherapy and anti-emetic therapy. The second (patient 14) described mildly distressing visual hallucinations. This patient had fever at the time of confusion which may well have exacerbated the adverse event. Symptoms resolved completely after 24h in both patients.

In general, the incidence of palmar/plantar syndrome was higher than expected and it is possible that Z.3HCL may have exacerbated this reaction. Abdominal pain experience was also higher than expected but this could be the result of enhanced monitoring in the trial setting. However, it was considered that the inclusion of Z.3HCL did not significantly increase the toxicity of the induction regime, with all individuals having average times for both engraftment and inpatient stay.

Table 3.1.4 Adverse Events to Z.3HCL. Table showing patient grade 3 toxicity reactions associated with drug combination

	Patient 4 200mg/ m^2 6h	Patient 6 300mg/ m^2 6h	Patient 7 300mg/ m^2 6h	Patient 8 400mg 3h	Patient 9 400mg 3h	Patient 11 400mg 3h	Patient 14 400mg 3h
Hallucinations				Grade 3			Grade3
Syncope	Grade 3			Grade 3			
Confusion/ vagueness		Grade 3					Grade 3
Short term memory loss			Grade 2				
Increased anxiety				Grade 3			
Hand tremor			Grade 1			Grade 1	
Abnormal dreams				Grade 3			
Lightheaded/ dizziness			Grade 2		Grade 2		
Vomiting				Grade 3			
Fatigue			Grade 2	Grade 3			

3.1.2.3 P-gp Expression

Blast cells isolated from 6 of the 16 (37.5%) patients assayed positivity for P-gp over-expression (MRK16 / Isotype ratio ≥1.1). These results are illustrated in Table 3.1.3.

3.1.2.4 R123 Efflux Assay

In the R123 assay, P-gp activity was completely inhibited in all 16 patients in the $CD56^+$ cells with a median percentage inhibition of 98% (range 73 - 111%) at 0.5 - 1h after start of Z.3HCL infusion. Similar inhibition of P-gp activity was also demonstrated at 2 - 3h (mean 90.5%, range 63-106%), with gradual loss in inhibition post 8h, as demonstrated by an increase in R123 MCF ratio (Figure. 3.1.2 A). This data is also represented on a per patient basis in Figure 3.1.2 B

Similarly, in $CD33^+$ cells in 6/10 patients where Z.3HCL induced P-gp efflux modulation was demonstrated, the mean inhibition was 85.25% (range 64-105.5%) at 0.5–1hr. The modulation by Z.3HCL in $P\text{-}gp^+$ and $P\text{-}gp^-$ patient cells are shown in Figure 3.1.2 C and E (mean) and D and F (individual). In the $P\text{-}gp^+$ cells, there was a decrease in mean modulation post 8h, similar to that seen in $CD56^+$ cells. However, this data is somewhat distorted by the fact that only one of the four in this group of $CD33^+$ cells (Patient 9) responded with an efflux profile similar to that seen in the $CD56^+$ cells (Figure 3.1.2 D).

Data were made available from the pharmacokinetic analysis performed off-site by Eli Lilly. The Z.3HCL plasma concentration-time curves for the three dosing patient groups relating to these data are shown in figure 3.1.3.A-C A direct

concentration-effect relationship between Z.3HCL plasma concentration and inhibition of R123 efflux in $CD56^+$ lymphocytes has been reported previously[318]. Based on this relationship all patients achieved plasma concentrations in excess of the IC_{90} value (figure 3.1.3).

The $CD56^+$ results can be further illustrated in Figure 3.1.4, where the data is displayed for the three dosing groups (A, B, C). This figure indicates that maximal P-gp inhibition was rapidly achieved and that inhibition was still active at approximately half-maximal at 24h irrespective of which dosing schedule were undertaken by the patient.

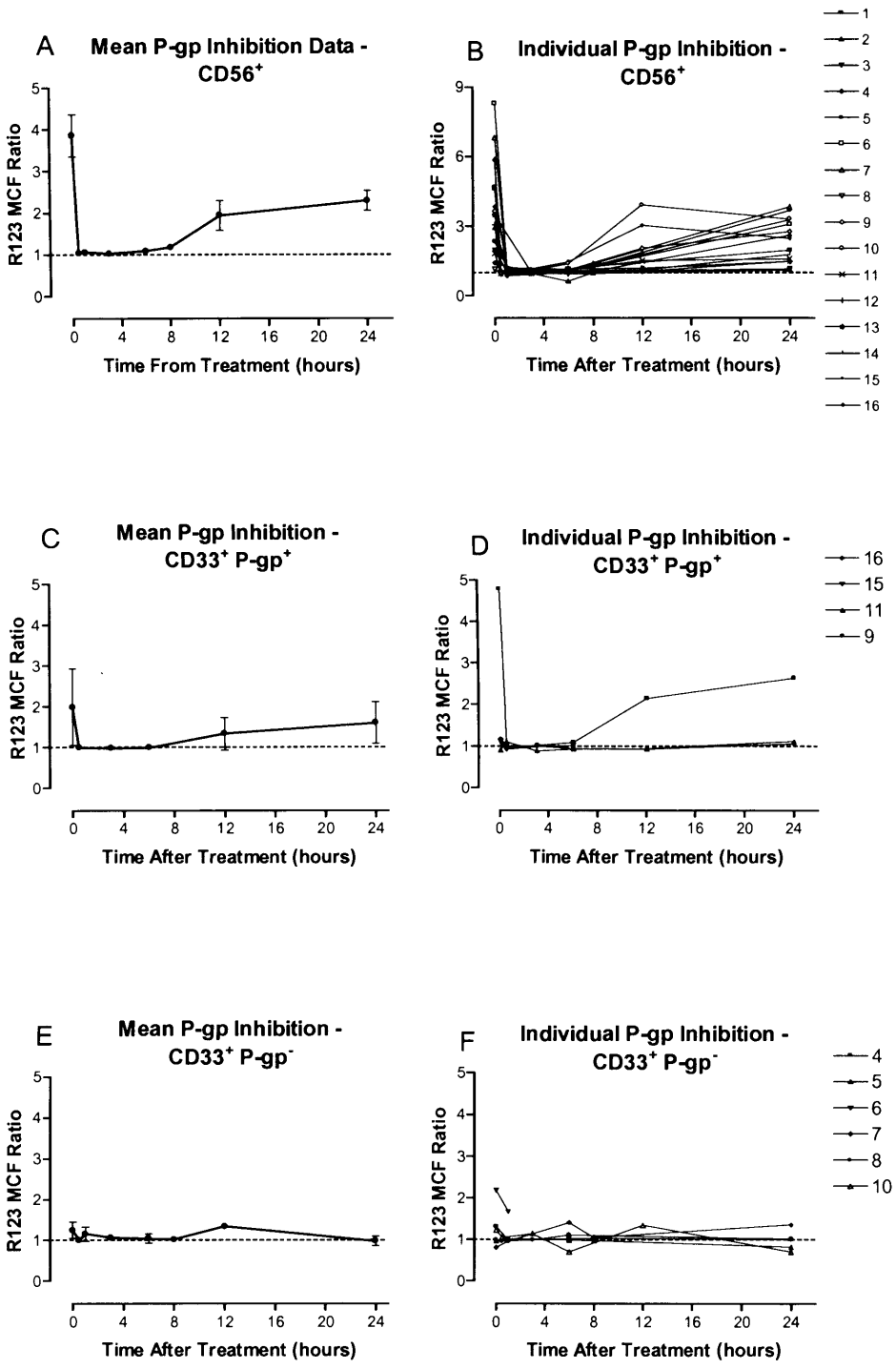


Figure 3.1.2 Results of the Pharmacodynamic R123 efflux assay: A - $CD56^+$ cells mean; B - $CD56^+$ cells individual; C - $CD33^+$ cells, $P\text{-}gp^+$ mean; D - $CD33^+$ cells, $P\text{-}gp^+$ individual; E - $CD33^+$ cells, $P\text{-}gp^-$ mean; E - $CD33^+$ cells, $P\text{-}gp^-$ individual.

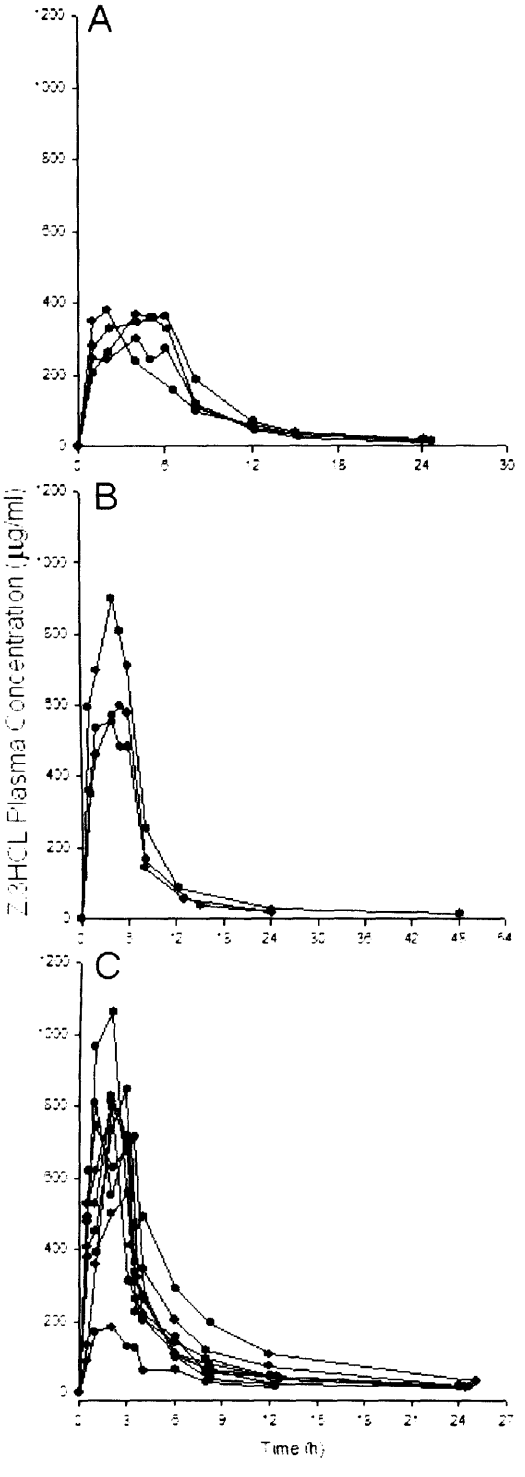


Figure 3.1.3 Plasma concentration-time curves for Z.3HCL in the 3 patient cohorts. A - 200mg/m^2/6h, B – 300mg/m^2/6h, and C – 400mg/3h.

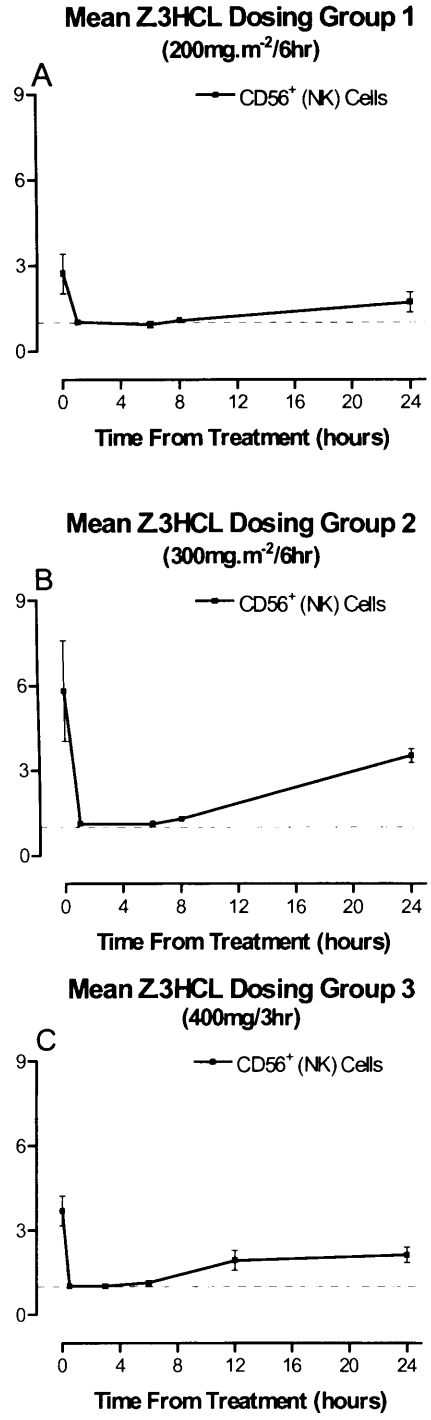


Figure 3.1.4. CD56+ cell response to P-gp inhibition by Z.3HCL, stratified by dosing group: A – 200mg.m^2 / 6hr; B – 300mg.m^2 / 6hr; C – 400mg / 3hr.

3.1.2.5 Calcein-AM Accumulation Correction Assays

Cells isolated from 9/15 patients and blast-gated using forward and side scatter showed functional P-gp calcein-AM efflux correction of MAF≥10 with Z.3HCL with a median MAF of 13.4. Similarly, 11/15 patients showed efflux correction with VPM with a mean MAF of 18.7. Seven patients exhibited an efflux correction by both compounds (Table 3.1.3). The median calcein-AM efflux correction was greater in those cells that over expressed P-gp (median MAF 37.9 for Z.3HCL and 35.4 for VPM). A significant correlation was found between P-gp expression with MRK16 and P-gp function by calcein-AM (Fig 3.1.6).

3.1.2.6 MTT Assay

The median IC_{50} levels for DNR show significant difference between cells modulated and not modulated *in vitro* with Z.3HCL (median IC_{50}= 246.9ngml^{-1} control vs 153.0ngml^{-1} +Z.3HCL, p= 0.012) implying that P-gp mediated efflux of DNR can be reversed by the addition of Z.3HCL. This effect was also seen for MIT, which is also a P-gp substrate (median IC_{50}= 141.1ngml^{-1} control vs 57.0ngml^{-1} +Z.3HCL, p= 0.037). There was no significant difference between the IC_{50} levels for ARA-C with and without Z.3HCL (median IC_{50}= 1571ngml^{-1} vs 2313ngml^{-1}, p= 0.313). Figure 3.1.5A

Further analysis showed that the patient cohort data could be subdivided and stratified as two separate groupings determined on functional P-gp performance (calcein-AM assay) shown in Figure 3.1.5B and P-gp expression (MRK16 assay) shown in Figure 3.1.5C.

Figure 3.1.5B shows the median IC_{50} results from the subset of patients (n= 9) that were shown to have a positive calcein-AM efflux function. The ARA-C results showed no significant drug sensitisation between the unmodulated and modulated group (2175ngml^{-1} and 2516ngml^{-1} respectively, p=0.3672). The DNR and MIT groups both show significant sensitisation between the median IC_{50}'s: DNR, 471.2ngml^{-1} unmodulated against 271.5ngml^{-1} modulated, (p=0.0371); MIT, 168.6ngml^{-1} unmodulated against 141.3ngml^{-1} modulated, (p=0.0273). These results are essentially similar to the results from the total median group.

Figure 3.1.5C shows the IC_{50} results from the subset of patients found to be P-gp^{+} by the MRK16 flow cytometry assay. Again, the pattern of sensitisation is similar to the efflux positive and total groups, only differing in the results for the MIT. Although showing a reduction in IC_{50} levels between the unmodulated and modulated groups (130 ngml^{-1} and 42.69ngml^{-1} respectively) this reduction was not found to be significant (p=0.1875). This could be due to the small sample size (n=4). The ARA-C showed no significant change (3303 ngml^{-1} unmodulated against 2516ngml^{-}1 modulated, p=0.3125) and the DNR did demonstrate significant sensitisation (160.3ngml^{-1} against 135.6ngml^{-1}, p=0.0313).

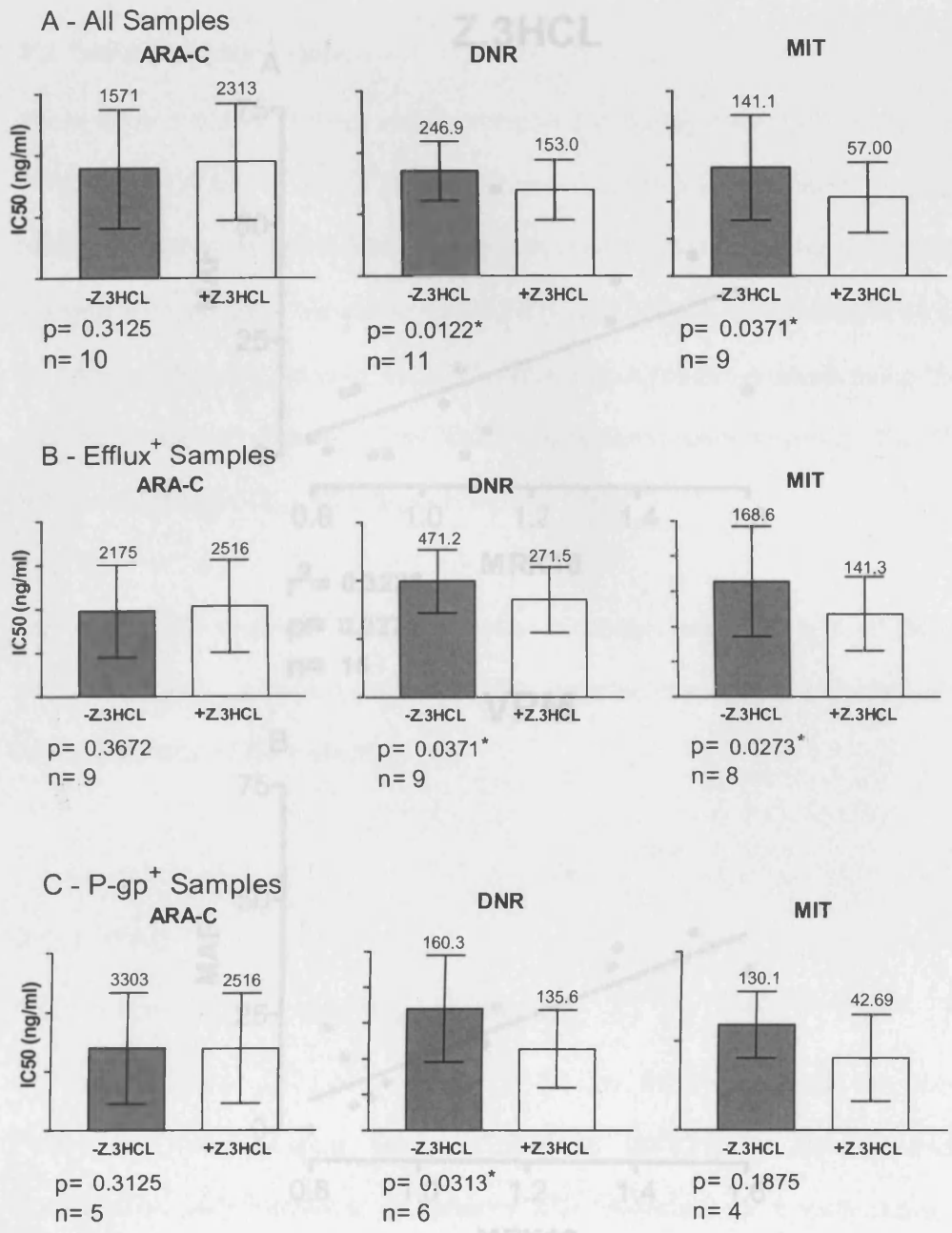


Figure 3.1.5 MTT Assays. The median IC_{50} values with and without Z.3HCL assayed against ARA-C, DNR and MIT. A - shows the median results from all the patients assayed; B - from calcein-AM efflux$^+$ patients; C - from MRK16 P-gp$^+$ patients. * Denotes a significant difference.

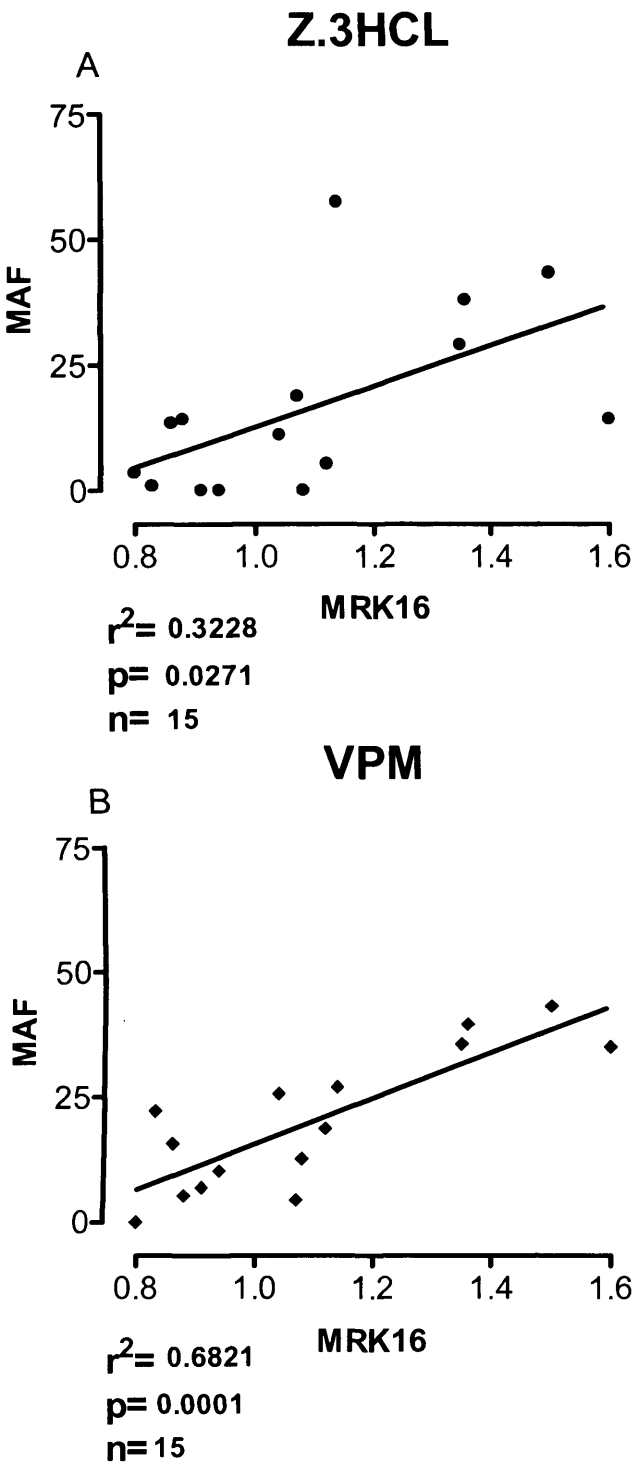


Figure 3.1.6 Correlation between P-gp expression by MRK16 MoAb and P-gp efflux function by calcein-AM accumulation assay in blast gated PBMC cells isolated from AML patients enrolled on the JTAN study. A shows the correlation for the P-gp inhibitor Z.3HCL, and B shows the correlation for VPM.

3.2 Solid Tumour Trials

There were two solid tumour trials participated in during the course of this study: JTAC and JTAM. For JTAC, peripheral blood samples were taken from patients based at sites in Glasgow (UK), Oxford (UK) and Amsterdam (The Netherlands), typically four samples over a period of 24 hours. These samples were shipped to the Royal Free Hospital by courier for pharmacodynamic analysis using the Eli Lilly R123 assay. For the JTAM study the patients were based at The Royal Marsden (UK) and UCL Middlesex Hospitals (UK).

Due to the limited amount of patient material, study design and lack of informed patient consent, no accessory *in vitro* studies were carried out on cells isolated from JTAC and JTAM patients.

3.2.1 JTAC

3.2.1.1 Introduction and Study Design

Designated JTAC – (B3T-MC-JTAC(d)) by Eli Lilly, the investigation was titled, A Phase 1 Dose Ranging Study of Z.3HCL (LY335979) Administered in Combination with Paclitaxel in Patients with Metastatic or Locally Advanced Cancer.

3.2.1.1.1 Objectives

The primary objective was to determine a dose of Z.3HCL that can be administered safely, with acceptable toxicity, in combination with paclitaxel.

The secondary objectives were to evaluate the toxicity of Z.3HCL administered in combination with paclitaxel; to compare the pharmacokinetics of paclitaxel with and without Z.3HCL; to determine the pharmacokinetics of Z.3HCL (and its metabolites) when administered in combination with paclitaxel; to identify a dose of Z.3HCL that could be used in combination with paclitaxel in future Phase 2 efficacy studies. The data from sixteen patients were collected during the course of our participation in the study.

3.2.1.1.2 Inclusion Criteria

Eligible patients were to be male or female of at least 18 years age exhibiting metastatic or locally advanced cancer not amenable to surgery or radiation. Prior surgery or radiation treatment should have been completed at least 3 weeks prior to study enrolment. Prior chemotherapy was allowed providing there had been no more than two regimes and that they had been completed 3 weeks before enrolment. Eligible candidates were to have a performance rating of 0 – 2 on the ECOG scale, adequate organ function, contraceptive measures to be taken for both females and males to minimise childbearing potential, and have an estimated life expectancy of at least 12 weeks.

3.2.1.1.3 Exclusion Criteria

Patients with unstable angina, myocardial infarction in previous six months, atrial or ventricular arrhythmia or congestive heart failure (CHF) were not considered eligible for enrolment. Other criteria for exclusion included: serious systemic infection; pregnancy or breast feeding; the use of any investigational agent within

four weeks prior to enrolment; diagnosis of a haematological malignancy; patients with seizure disorders; history of brain metastases.

3.2.1.1.4 Dosing

The Z.3HCL was administered orally, with seven dose escalation cohorts in total. The doses were given 12hr apart for between 2 and 7 days, except for cohort 1, where the dosing was administered every 8hr for 10 days. The dosing schedule for Z.3HCL started at 100mg/m^2 in cohort 1, increasing to 200mg/m^2 for cohort 2, 300mg/m^2 for cohorts 3 and 4, 250mg/m^2 for cohort 5. Cohorts 6 and 7 received 450mg and 500mg total respectively. The paclitaxel dose was 175mg/m^2 for cohorts 1 to 5, and 225gm/m^2 for cohorts 6 to 7.

3.2.1.1.5 Patient Demographics

There were sixteen patients studied: 13 female and 3 male, with a median age of 55 years (range 31 – 77 years). There were 6 lung cancers (5 non-small cell lung cancer (NSCLC), 1 adenocarcinoma); 3 breast cancers (2 ductal, 1 lobular); 3 ovarian cancers; 1 endometrium; 1 melanoma; 1 oesophagus; and 1 stomach cancer. The median time from diagnosis was 15 months (range 2 – 166 months) (Table 3.2.1).

3.2.1.1.6 Pharmacodynamic Assay

The Eli Lilly protocol R123 accumulation flow cytometry assay was used to measure the degree to which Z.3HCL inhibited the P-gp efflux function in $CD56^+$

NK cells isolated from patient peripheral blood, sampled during the course of Z.3HCL administration.

3.2.1.2 Results

3.2.1.2.1 Clinical Data

The available clinical data made available by Eli Lilly shows that of the 16 patients involved with the *in vitro* study at the Royal Free, 10 patients achieved a best response of stable disease (SD). One patient showed progressive disease, while a further 5 were not evaluated. No analysis was performed on the clinical benefits of P-gp modulation in these patients (Table 3.2.1).

3.2.1.2.2 Pharmacodynamic Assay

In the $CD56^+$ flow cytometry assay 14 / 16 patients showed inhibition of R123 efflux in response to the oral Z.3HCL. This inhibition was denoted by a greater than two-fold reduction in percentage efflux from the 0h time sample (Figures 3.2.1 and 3.2.2).

3.2.1.2.3 Adverse Events

Toxicity data was not made available for individual patients and therefore cannot be broken down to apply to the patients whose samples were analysed at the Royal Free. However Eli Lilly report that for the study overall, non-haematological toxicities associated with the modulator combination therapy were mainly neurological and included hallucinations, alopecia, myalgia, fatigue, neuropathy, visual disturbances, tremor, and ataxia[319].

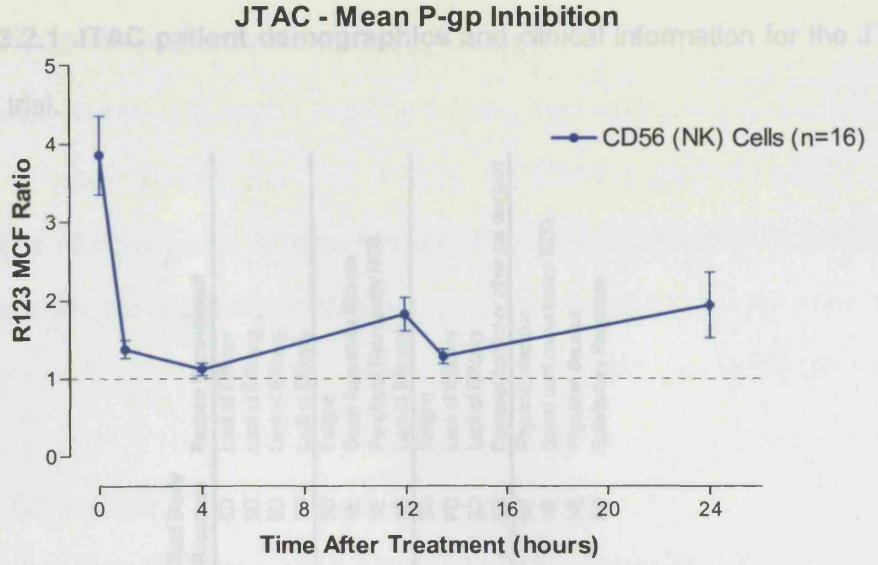


Figure 3.2.1 Results of the R123 Accumulation Assay for JTAC Clinical Trial. Demonstrating the mean P-gp inhibition in $CD56^+$ isolated from 16 patient samples.

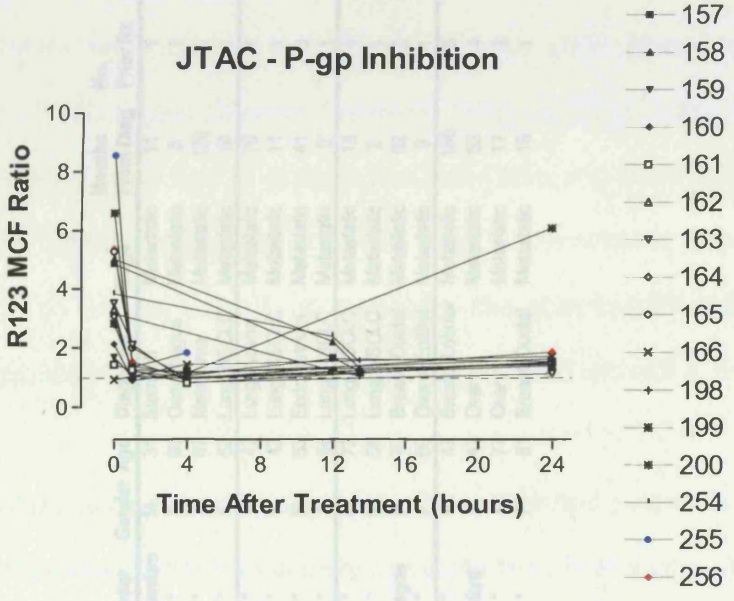


Figure 3.2.2 Results of the R123 Accumulation Assay for JTAC Clinical Trial. Demonstrating the individual P-gp inhibition in $CD56^+$ isolated from 16 patient samples.

Table 3.2.1 JTAC patient demographics and clinical information for the JTAC clinical trial.

Patient		Center	Gender	Age	Diagnosis	Stage	Months From Diag	No. Prior Rx	Z.3HCL Dose (mg/m2)	Paclitaxel Dose (mg/m2)	Best Study Response	Reason Discontinued
1	157	Amsterdam	M	31	Stomach	Metastatic	11	1	239	174	SD	Lack of Efficacy
2	158	"	F	60	Oesophagus	Metastatic	8	1	238	177	SD	Lack of Efficacy
3	159	"	F	67	Melanoma	Metastatic	120	1	237	226	SD	Lack of Efficacy
4	160	"	F	52	Lung, NSCLC	Metastatic	10	1	268	225	SD	Lack of Efficacy
5	161	"	F	47	Lung, Adeno	Metastatic	70	1	300	229	SD	Fatigue
6	162	"	F	43	Lung, NSCLC	Metastatic	11	1	286	225	NE	Death from study disease
7	163	"	F	53	Endometrium	Metastatic	41	3	272	225	NE	Peripheral Neuropathy NOS
8	164	"	F	54	Lung, NSCLC	Metastatic	9	1	258	225	SD	Lack of Efficacy
9	165	"	M	72	Lung, NSCLC	Metastatic	15	1	250	175	SD	Fatigue
10	166	"	M	56	Lung, NSCLC	Metastatic	2	1	303	170	PD	Lack of Efficacy
11	198	Glasgow	F	70	Breast, Ductal	Metastatic	92	2	260	175	SD	Lack of Efficacy
12	199	"	F	69	Ovary, Epithel	Metastatic	8	1	316	225	SD	Personal conflict or other pat decision
13	200	"	F	47	Breast, Lobular	Metastatic	166	5	-	226	NE	Physician decision
14	254	Oxford	F	47	Ovary	Metastatic	52	4	250	175	NE	Spinal cord compression NOS
15	255	"	F	77	Ovary	Metastatic	17	2	347	222	NE	Physician decision
16	256	"	F	61	Breast, Ductal	Metastatic	15	1	298	173	SD	Satisfactory Response

3.2.2 JTAM - Introduction and Study Design

Designated JTAM – (B3T-MC-JTAM) by Eli Lilly, the investigation was entitled, A Phase 1 Dose Ranging Study of Z.3HCL (LY335979) plus Doxorubicin and Z.3HCL (LY335979) plus Doxorubicin and Cyclophosphamide in Patients with Metastatic or Locally Advanced Cancer.

3.2.2.1 Objectives

The primary objective of this study was to determine a dose of Z.3HCL that could be administered safely and with acceptable toxicity in combination with doxorubicin (DXR) alone (dose schedule part A), and with doxorubicin plus cyclophosphamide (CPM) (dose schedule part B) in patients with metastatic or locally advanced cancer. The secondary objectives were to evaluate the toxicity of Z.3HCL when administered in combination with DXR alone, and when administered with DXR plus CPM; to determine the pharmacokinetics of Z.3HCL and its metabolites alone and in combination with DXR, and DXR plus CPM; to determine the pharmacokinetics of DXR and its metabolite doxorubicinol (DXRL), with and without Z.3HCL; to determine the pharmacokinetics of DXR and DXRL administered in combination with CPM with and without Z.3HCL.

Due to difficulties encountered implementing the study and problems with slow enrolment Eli Lilly decided to prematurely terminate the JTAM study. All patients enrolled participated in dosing schedule part A (DXR & Z.3HCL). Dosing schedule part B (DXR & CPM & Z.3HCL) was not implemented.

3.2.2.2 Inclusion Criteria:

Patients to be enrolled had metastatic or locally advanced cancer, not amenable to surgery or radiation. Any prior surgery or radiation treatment was to have been completed at least four weeks prior to study enrolment. Prior chemotherapy treatment was allowed only if the candidate had received no more than 2 courses of therapy and that they had been completed at least 3 weeks before enrolment.

Prospective patients were to have had a performance rating of 0 – 2 on the ECOG scale with adequate organ function and an estimated life expectancy of at least 12 weeks. Patients were also to show a MUGA (Multiple Gated Acquisition) scan or echocardiogram with an ejection fraction of $\geq$50%, and to utilise appropriate contraceptive measures for both females and males to minimise childbearing potential.

3.2.2.3 Exclusion Criteria

Patients were not considered eligible for enrolment if they'd had use of any investigational agent within 4 weeks prior to enrolment or had a >300mg/m doxorubicin cumulative lifetime exposure. Other exclusion criteria included: a serious systemic infection; pregnancy or breast feeding; unstable angina, myocardial infarction in previous 6 months, atrial or ventricular arrhythmia or congestive heart failure (CHF). Patients were also excluded if they had a diagnosis of a haematological malignancy; concomitant hormonal or other anti-cancer therapy; known or suspected CNS metastases; or any previous involvement with a Z.3HCL trial.

3.2.2.4 Patient Demographics

Of the 11 patients enrolled that had samples analysed, 4 patients were diagnosed with sarcoma (3 metastatic, 1 locally advanced), 3 patients with lung cancer (1 bronchial, 1 large cell (LCLC), and 1 adenocarcinoma), 1 with adenoid cyst carcinoma, 1 colon cancer, 1 liver cancer, and 1 patient diagnosed with oesophageal cancer. All the non-sarcoma tumours were diagnosed as metastatic except the bronchial lung cancer and the LCLC which were locally advanced (table 3.2.2). The median time from diagnosis was 10.5 months (range of 2 – 32 months) and the median number of previous therapies was 2 (range of 1 – 3).

3.2.2.5 Dosing

The Z.3HCL was administered as an oral capsule, with an initial dose of 200mg every 12 hours. The escalation of Z.3HCL was in 100mg steps until the achievement of the maximally tolerated dose (MTD). The median Z.3HCL dose was 400mg/m^2 (range 200 – 500mg/m^2) and the DXR dose was 75mg/m^2 (no escalation).

3.2.2.6 Study Amendment

There were originally planned to be 2 parts to the dose regimen, schedule A and schedule B, but because of the early termination of the study, only schedule A was implemented. For dosing schedule A, on cycle 1, 3 and any subsequent cycles, Z.3HCL was given on Day 1 for a total of 2 doses (12 hours apart) with

75mg/m^2 DXR given as 30 minute infusion 2 hours after the first dose of Z.3HCL. For cycle 2, 75mg/m^2 DXR was given alone on day 1.

3.2.2.7 Sample Schedule

Blood samples were taken at 0, 2, 4 and 24h after the commencement of Z.3HCL therapy. The first three samples were sent via courier at the end of the first day, and the remaining 24h sample sent on the second day straight after being taken. The samples were processed and analysed immediately upon receipt at the Royal Free Hospital.

3.2.3 Results

3.2.3.1 Pharmacodynamic Assay

It can be seen from figure 3.2.3 that P-gp efflux function, as demonstrated using the Eli Lilly R123 flow cytometry assay, was completely and rapidly inhibited in 9 / 11 assessable patients. Significant inhibition was denoted by a greater than 2 fold reduction in R123 MCF ratio. This data is also shown for individual patients in Table 3.2.4.

3.2.3.2 Clinical Results

Data provided by Eli Lilly report that 3 / 11 patients demonstrated a study best result of stable disease (SD), 5 with progressive disease (PD), and 3 patients that were unevaluated (NE). No modulator benefit analysis was conducted.

Table 3.2.2 JTAM patient demographics and clinical information for the JTAM clinical trial.

	Patient	Centre	Gender	Age	Diagnosis	Stage	Months From Diag	No. Prior Rx	Z.3HCL Dose (mg/m2)	DXR Dose (mg/m2)	Best Study Response	Reason Discontinued
1	8001	Royal Marsden	M	63	Sarcoma	Metastatic	12	2	200	75	PD	Physician Decision
2	8003	Royal Marsden	M	54	Sarcoma	Locally Advance	15	2	200	75	SD	Physician Decision
3	8004	Royal Marsden	F	57	Sarcoma	Metastatic	-	2	200	75	PD	Physician Decision
4	8005	UCL Middlesex	M	53	Sarcoma	Metastatic	2	1	400	75	PD	Physician Decision
5	8026	Royal Marsden	M	67	Lung, Bronchial	Locally Advance	11	2	400	75	NE	Evidence of diminishing cardiac function
6	8027	UCL Middlesex	F	58	Liver	Metastatic	8	2	400	75	PD	Death from study disease
7	8028	UCL Middlesex	M	65	Lung, Lg Cell	Locally Advance	10	2	400	75	SD	Hypoaesthesia
8	8030	UCL Middlesex	M	48	Lung, Adeno	Metastatic	23	3	500	75	NE	Pancytopaenia
9	8031	UCL Middlesex	M	52	Colon	Metastatic	32	3	500	75	SD	Evidence of diminishing cardiac function
10	8032	UCL Middlesex	M	63	Oesophagus	Metastatic	4	2	500	75	PD	Death from study disease
11	8034	UCL Middlesex	M	36	Adenoid Cyst Ca	Metastatic	8	1	500	75	-	Neutropenia

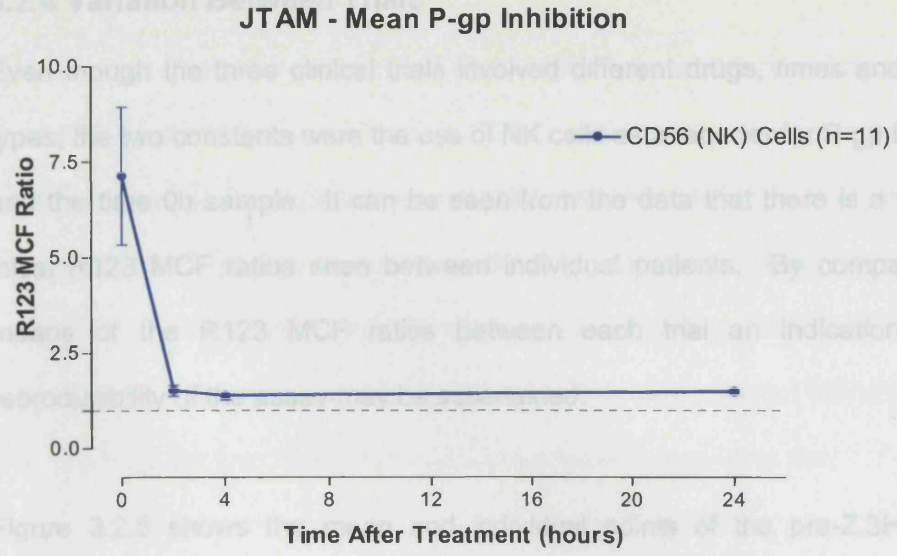


Figure 3.2.3 Results of the R123 Accumulation Assay for JTAM Clinical Trial. Demonstrating the mean P-gp inhibition in $CD56^+$ isolated from 11 patient samples.

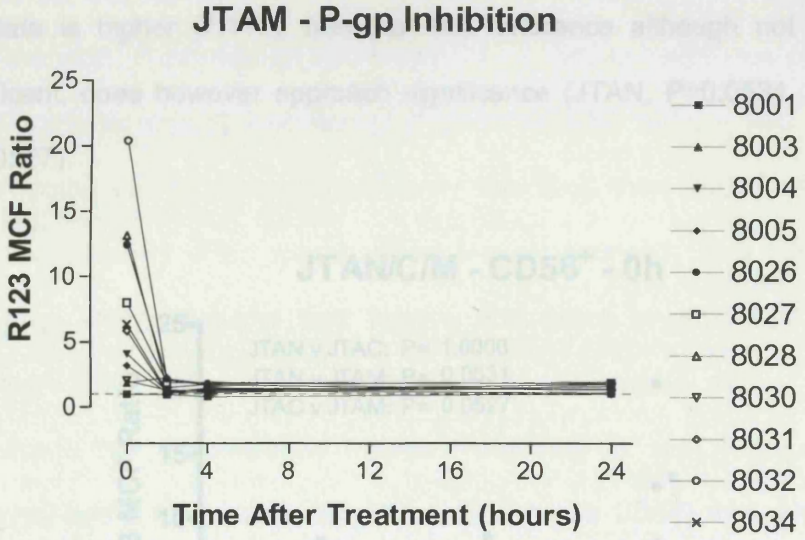


Figure 3.2.4 Results of the R123 Accumulation Assay for JTAM Clinical Trial. Demonstrating the individual P-gp inhibition in $CD56^+$ isolated from 11 patient samples.

3.2.4 Variation Between Trials

Even though the three clinical trials involved different drugs, times and tumour types, the two constants were the use of NK cells as a reporter for P-gp inhibition and the time 0h sample. It can be seen from the data that there is a range of initial R123 MCF ratios seen between individual patients. By comparing the means of the R123 MCF ratios between each trial an indication of the reproducibility of the assay may be ascertained.

Figure 3.2.5 shows the mean and individual points of the pre-Z.3HCL (0h) samples for each of the three trials. It can be seen that the mean and spread of the data for JTAN and JTAC are very similar (Mean = 3.852 for both) and this is reflected in the result of the t-test performed between the two (P=1.000). The variation between patients in JTAM is by contrast much larger, and the mean of the data is higher (7.117), and that this difference although not statistically significant, does however approach significance (JTAN, P=0.0531, and JTAC, P=0.0527)

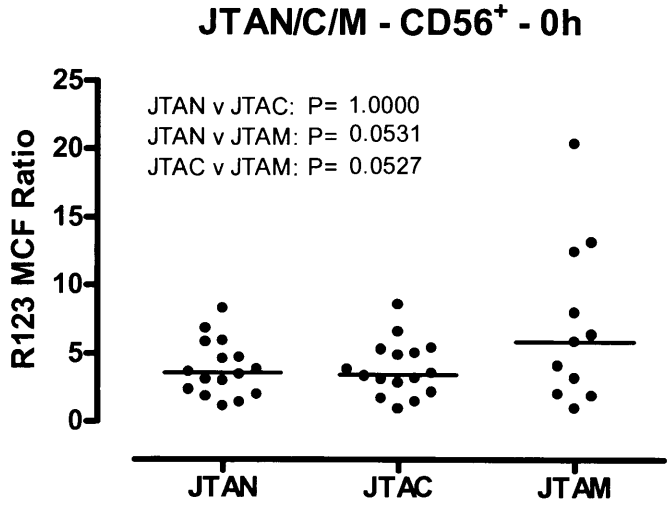


Figure 3.2.5 Inter-Trial Assay Variability.

3.3 Discussion

The pharmacodynamic data from all three trials show a corresponding pattern of *in vivo* P-gp inhibition in circulating $CD56^+$ cells, with almost complete efflux inhibition being demonstrated very rapidly after commencement of treatment, regardless of the dosing regimen or route of administration. It can be seen that when only a single dose regimen was implemented P-gp inhibition begins to diminish at 8-12h, but still retaining approximately half-maximal efficacy at 24h. The two-dose regimen demonstrated a maximal inhibition up to 24h.

3.3.1 JTAN AML Trial

The R123 accumulation flow cytometry data clearly show Z.3HCL to be a significant P-gp inhibitor in patients with AML. R123 efflux from $CD56^+$ cells was completely and rapidly inhibited in all the patients studied, often by 0.5 hours from the start of infusion of the modulator. A similar trend was seen for the $CD33^+$ cells confirming inhibition of P-gp in the AML cells *in vivo*. However, $CD33^+$ cells tend to express significantly less P-gp than $CD56^+$ cells and the observed inhibitory effect was therefore less pronounced. It is this inherently high P-gp expression and their relative abundance in peripheral blood that qualifies $CD56^+$ cells as a convenient *in vitro* surrogate marker for use in fluorescent dye accumulation assays measuring *in vivo* P-gp modulation. However, some AML blasts are known to express CD56, and since in most cases blasts cells express a much lower level of P-gp than NK cells, this may have had the effect of reducing the observed sensitivity of the assay. In a Phase II trial with Z.3HCL, *in vitro* studies on bone marrow myeloblasts also demonstrated successful modulation of P-gp efflux[320]. On hindsight, inclusion of

CD45 as a co-marker would have made the assay more robust, since this would have allowed for more accurate gating of the blast cell population.

The calcein-AM accumulation assay showed Z.3HCL to be as almost as effective as VPM at inhibiting functional P-gp efflux *in vitro*. Although more patients showed calcein-AM efflux correction with VPM than with Z.3HCL, 5/6 P-gp$^+$ patients exhibited efflux correction with both modulators. The higher correction and better correlation by VPM may be attributed to its ability to inhibit other drug transporters including, to some extent, MRP1 while Z.3HCL solely inhibits P-gp modulated efflux. This lack of selectivity by VPM can however, result in deleterious changes to the pharmacokinetics of co-administered drugs, a problem not associated with Z.3HCL. The clinical relevance of MRP1 expression as a significant resistance mechanism in AML is still being debated but its expression in this cohort of samples appear not to contribute to the results.

The drug sensitivity studies using the MTT assay show that co-incubation with Z.3HCL *in vitro* renders the AML cells from patients more sensitive to DNR and MIT. DNR is a recognised substrate for P-gp and MIT can be effluxed by P-gp and the related half-transporter, BCRP. Both drugs would therefore be removed from the cell by P-gp unlike ARA-C, which is not a substrate and consequently there is no sensitisation to ARA-C by the addition of Z.3HCL. The reduction in the IC_{50} for DNR confirms that Z.3HCL treatment in this cohort of patients would have inhibited the efflux of the infused DNR in AML cells thereby contributing to cell death and consequent treatment response.

The results suggest that Z.3HCL is well tolerated in combination with standard induction agents DNR and ARA-C. The main toxicities manifested as neurological events. This is in keeping with the fact that P-gp is expressed in brain endothelial cells and may represent direct drug effect, concomitant cytotoxic drug effect or effects related to inability to efflux naturally occurring toxins or metabolites. There is however, no apparent correlation with plasma drug concentrations of Z.3HCL or with the degree of P-gp inhibition as all patients achieved maximal inhibition in the R123 assay. Hence the possible mechanisms for this remain unknown[318].

An additional adverse event with the use of P-gp modulators *in vivo* is the enhanced toxicity of the co-administered cytotoxic agent. Although second generation P-gp inhibitors such as PSC833 and CSA are characterised by enhanced activity against P-gp, they also affect the pharmacokinetics of the cytotoxic agents with which they were administered. However, Z.3HCL has been shown to have minimal effect on doxorubicin toxicity or pharmacokinetics as shown by a phase I study in patients with advanced malignancies[321].

Previous studies have observed that since the bulk of the AML cells do not over express P-gp, it is possible that P-gp$^+$ clonogenic AML blast cells may form a seed reservoir of resistant cells that contribute to conventional treatment failure. It is further suggested that this minor population should be the target for P-gp modulation as indeed was done in this study by the infusion of Z.3HCL on the third and fifth day post initial treatment with DNR and ARA-C. It was assumed that this would have eliminated the sensitive pool of AML cells to be followed by an effect on resistant cells consequent to P-gp modulation. The benefits of this

modulation however may not be seen in a CR but in the DFS or in the time to relapse[322]. However, studies by van der Pol *et al* suggest that the emergence of minimal residual disease (MRD) in AML is not by selection of pre-existing resistant subpopulation cells[191].

These data suggest that Z.3HCL shows promise as a clinically useful adjuvant due to its high specificity, potency and lack of deleterious pharmacokinetic interactions. Large scale randomized trials are warranted to assess the clinical efficacy of Z.3HCL in AML. Z.3HCL in combination with the C-AM and MTT assays is useful in ascertaining specific P-gp functional resistance. Results of this trial in AML have been recently published by Gerrard *et al* in Haematologica[323], and by Callies *et al* in Cancer Chemother Pharmacol[318].

3.3.2 JTAC Solid Tumour Trial

The focus of this study was the clinical dosing and safety data, which was analysed offsite by Eli Lilly. The pharmacodynamic assay showed that $CD56^+$ cells isolated from 14 of the 16 patients assayed showed a rapid greater than two-fold reduction in P-gp efflux in response to oral Z.3HCL.

It can be seen from figure 3.2.2 that there was a wide range of initial points for the individual inhibition ratio graphs, possibly reflecting the wide range of variables involved, including variability between the centres concerned, differences in acquiring the blood samples, between patient disease, and the length of time the samples were in transit before analysis. When taken together,

these factors may help explain the wide range of heterogeneity in the starting assay. The oral route of Z.3HCL administration may also contribute a degree of variation due to possible differences in bioavailability.

None of the patients involved were assayed to determine P-gp over-expression in their tumour tissues, although all of the tumour types in question have been shown to be affected by P-gp mediated MDR[324-326].

Overall, the pharmacodynamic results do indicate that oral Z.3HCL at all of the dosage regimens implemented appears to be an effective inhibitor of P-gp mediated efflux and therefore may contribute positively as an adjuvant to chemotherapy where P-gp associated MDR has been indicated as a obstacle to successful treatment. The pharmacokinetic data from this trial was published by Callies *et al*[279].

3.3.3 JTAM Solid Tumour Trial

As with the previous solid tumour trial (JTAC), the primary focus of this investigation was the safety and pharmacokinetic data, which were analysed off-site by Eli Lilly. The pharmacodynamic analysis of the surrogate $CD56^+$ cells showed that overall there was a rapid and prolonged response to the oral administration of Z.3HCL, with cells isolated from 9 of the 11 patients showing significant efflux inhibition.

It can be seen from Figure 3.2.4 that, as seen previously, there was a wide range of initial efflux ratios indicating some degree of variability, which could be attributed to the inherent number of variables involved. These include the differences in patient condition, sample location and transport. All the samples subsequent to the initial pre-dose samples show a level of uniformity in response to Z.3HCL, maintaining an effective and prolonged inhibition of efflux function. Pharmacokinetic data from this trial was published by Callies *et al*[278].

It was also observed that there was a wider range of pre-dose (time=0h) $CD56^+$ R123 efflux start points for patient samples from the JTAM trial compared with samples from the JTAN and JTAC, which were virtually identical (Figure 3.2.5). No obvious explanation for this finding can be offered other than some unidentified source centre specific variable. All three trials ran concomitantly with overlapping sampling durations, with JTAC starting first, then JTAM and then JTAN. All patient samples for JTAN were sourced at the Royal Free, while all samples for JTAC and JTAN were same-day couriered from external sites. It is worth noting, however, that only 3 of the 11 JTAM samples fell outside of the range observed for the other two trials, and these 3 samples came from 2 different centres and were not received contiguously. Since the statistical analysis found no actual significant difference, therefore it must be considered that the observed difference may well be stochastic in origin.

In summary, three phase I clinical trials of Z.3HCL were conducted: JTAC, JTAM, and JTAN. The first two trials were limited in their scope for investigating the efficacy of Z.3HCL in the clinical setting, primarily because they were

conducted on patients with solid tumours, and secondarily because the focus of these investigations (and indeed JTAN as well) was to facilitate the acquisition of dose range and drug combinational safety data. The third trial, JTAN, was more amenable to the attainment of MDR related data and the monitoring of inhibition efficacy, notably in surrogate cells, but also in malignant cells. However, the sample size of patients was too small to enable a realistic evaluation of benefit arising from the use of Z.3HCL in the clinical setting.

In the clinical setting, Z.3HCL is shown to be a relatively safe and effective inhibitor of P-gp efflux function, both as an oral (JTAC and JTAM) or IV treatment (JTAN). These data add weight to the argument for it's inclusion in further efficacy studies as an adjuvant for leukaemia and cancer chemotherapy where P-gp has been shown to be an adverse prognostic signifier.

4. *In Vitro* Studies – Multidrug Resistance

4.1 General Introduction

MDR, and in particular P-gp over-expression has been shown to be a risk factor that impacts negatively upon the chemotherapeutic treatment of cancer in general, and haematological malignancies in particular. The routine monitoring of MDR levels in leukaemia patients using both quantitative and qualitative methods has been established and implemented by our laboratory at the Royal Free Hospital. Z.3HCL, because of its proven selectivity and potency, was utilized as a component of the assays used in this analysis along side the established P-gp inhibitor verapamil (VPM), thereby enabling further examination of its character and ability as a P-gp inhibitor in patient material and *in vitro* cell line studies.

4.1.1 Patient Sample Material

Cells from patient peripheral blood and bone marrow samples were tested for the expression of MDR proteins using monoclonal antibodies and for functional P-gp efflux using the calcein-AM assay. MTT drug sensitivity assays were performed where appropriate and where sufficient patient material was available. The P-gp inhibiting efficacy of Z.3HCL was evaluated in the different disease types in order to ascertain the suitability of Z.3HCL as a P-gp modulatory agent for future clinical trials in these diseases. Of initial interest was the mature B-lymphocyte malignancy, multiple myeloma, and although this study has shown Z.3HCL to be an efficient reverser of P-gp in tumour cells isolated from myeloma patients, the inherent difficulties in obtaining sufficient numbers of pure tumour

cells from small volumes of typically low-cellular marrow samples frustrated most attempts to conduct drug sensitivity assays.

4.1.2 Calcein-AM Data

The calcein-AM dye accumulation assay yielded the largest number of data, and is conceivably the more useful of the assays performed in indicating the MDR status of a sample because it demonstrates P-gp function rather than expression, although the two are strongly correlated (Figure 4.1).

4.1.3 Expression Stratification

To give a realistic indication of the P-gp status of an individual patient sample at least two parameters should be measured, and in the case of this study, a functional P-gp (calcein-AM assay) and a quantitative P-gp expression assay (MRK16 MoAb) were utilized. This strategy allows for the stratification of patient samples into three expression profiles: negative, intermediate and high. For a patient sample to be classified as negative, both tests for P-gp would be below the threshold for positivity. For a patient sample to be intermediate one or other of the two assays would be positive, and a patient sample to be classed as high, both assays would prove positive.

4.1.4 MTT IC_{50} Data

The MTT drug sensitivity assay when used with and without Z.3HCL is a useful indicator of the significance of P-gp in the MDR character of a sample cell

population. Non-parametric, one-tailed Mann-Whitney tests were performed between the median of each data set in order to determine any significant sensitisation to the drugs by the presence of Z.3HCL.

Typically, three drugs were utilized in the assay, and these were chosen to reflect drugs in clinical use for the treatment of each disease. As seen in Chapter 3, ARA-C, DNR and MIT were used in the MTT assay for cells isolated from AML patients. DNR and MIT are P-gp substrates and ARA-C is not.

The MTT data for CLL patient cells included only one P-gp effluxed drug, DNR. Chlorambucil (CHL) and Fludarabine (FLU) are two of the main first line drugs used in the treatment of patients with CLL and neither of these drugs are a substrate for P-gp mediated efflux. Although a trend is seen in the combined IC_{50} data towards a sensitisation in the presence of Z.3HCL, none of the results proved to be significant at the 95% confidence level, even for DNR in the P-gp efflux selected group. This again may be a consequence of the low sample numbers. Less priority was given to the comparative study of CLL patients with Z.3HCL because of the fact that most of the drugs used in its treatment are not P-gp efflux substrates.

Due to the difficulties associated with the attainment of sufficient numbers of viable MM plasma cells, no meaningful MTT results were obtained for these samples.

4.2 Results

4.2.1 AML Patient Samples

Over a period of three years AML patient samples were collected, prepared and analysed (see Appendix 1). Of these, 55 samples (26 PB, 29 BM) from 48 patients yielded useful results for both the calcein-AM functional assay and MRK16 quantitative P-gp assay. There were 35 males and 13 females and the median age of the patients was 60 years (range of 11 to 82 years), with a median WBC of 12.2 x 10^9L^{-1} (range of 0.7 to 242.0 x 10^9L^{-1}) and a median bone marrow blast count (where available) of 79% (range of 10 to 100%).

There were 29 presentation samples from *de novo* patients, 8 samples from previously relapsed patients and 9 samples from patients following treatment, with another 9 samples where the patient status was unknown. There were 6 patients from whom we received both an initial and a follow-up sample (including one patient who had 2 follow-up samples), with a median interval time of 7.7 months. There were 4 *de novo* samples with a follow-up at post treatment, 1 *de novo* sample with a relapse follow-up sample, and 1 relapsed sample with a follow-up at post-treatment. Of the first 4 *de novo* sample group, 3 were positive for P-gp function (MAF) at presentation and 2 remained positive post treatment, one patient was positive for P-gp expression at presentation, and 3 were positive post treatment. The remaining two patients were both positive for P-gp function and P-gp expression at both initial and follow-up time points.

Therapeutic response data was available for 33 patients, with 23 achieving a complete haematological response (CR), 7 who did not attain a CR (NR), and 3 showing progressive disease (PD).

Thirty patients had cytogenetic data available and were stratified into three prognostic risk groups[197]: Favourable (F), Intermediate (I) and Adverse (A). Two patients were classified as F, 26 as I, and 2 as A. These groups were too small to enable any meaningful correlative statistics, but a descriptive table detailing relative median WBC, age and MDR status is given in Table 4.1.

Table 4.1 Cytogenetic Risk Groups in AML patient cohort.

	No.	%	Age	WBC	MAF	P-gp
F	2	6.7%	44.5	27.15	15.70	1.23
I	26	86.7%	55	9.8	10.01	0.99
A	2	6.7%	48	11.05	21.18	1.11

Risk Group: F= Favourable; I= Intermediate; A= Adverse. WBC = median white cell count

4.2.1.1 AML MDR Assay Correlation

Figure 4.1 shows the correlation between P-gp expression, as measured by the MRK16 flow cytometry assay, and MDR efflux function, as measured by the calcein-AM accumulation assay. The latter assay utilized Z.3HCL (Figure 4.1A) and VPM (Figure 4.1B) as the P-gp inhibitor, allowing comparison between the two and thus enabling a measurement of the efficacy of Z.3HCL in the *in vitro* environment.

It can be seen that for both Z.3HCL and VPM calcein-AM assays the correlations with P-gp were highly significant ($P<0.0001$ for both), but probably due to the heterogeneous nature of the disease the r^2 (coefficient of determination) is relatively low (0.4224 for Z.3HCL and 0.4813 for VPM).

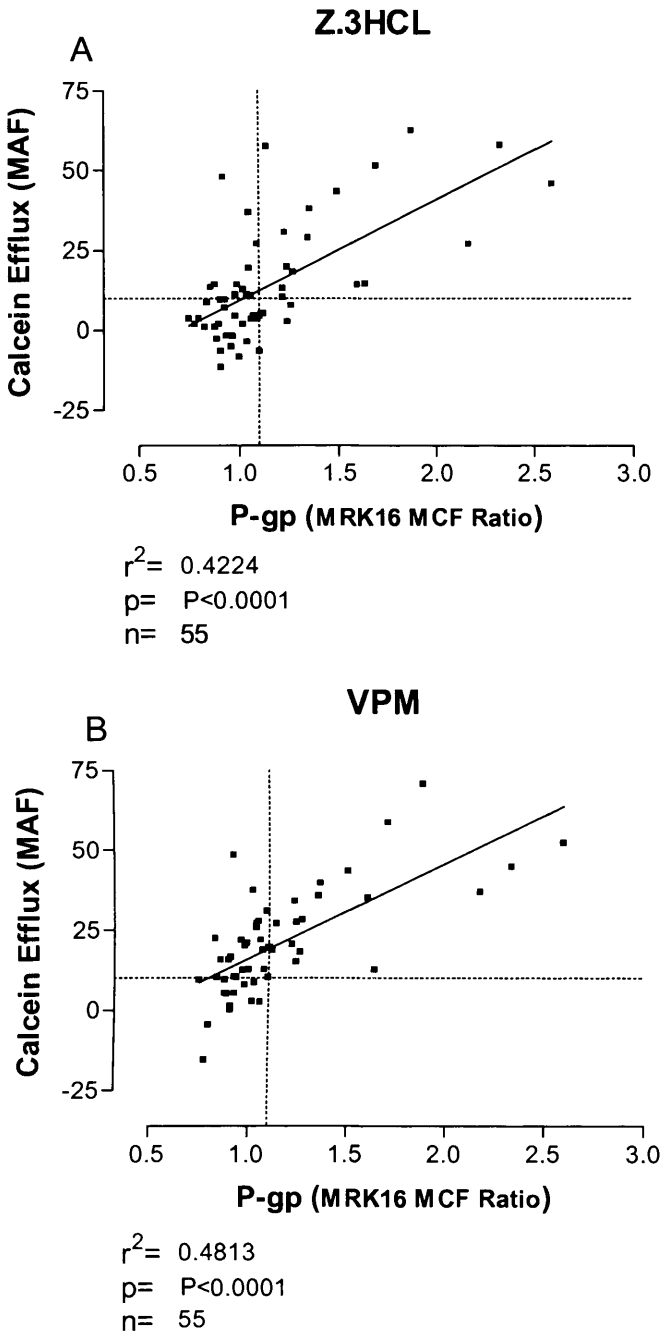


Figure 4.1 P-gp Expression and Calcein-AM accumulation correlation in AML. These graphs show the correlation between P-gp expression measured by MRK16 MoAb and functional Calcein dye efflux inhibition, as measured by MAF. A p-value is shown to indicate significance, and r^2 denotes the coefficient of determination.

4.2.1.2 AML MDR Assays

Table 4.2 shows the basic results for positivity in the two MDR assays (P-gp expression and efflux function). Figure 4.2 illustrates the results from the MRK16 P-gp expression and calcein-AM functional assays, with the intention of stratifying the patient samples into one of four MDR categories: positive (+), double positive (+/+), negative (-), and double negative (-/-).

Table 4.2 Basic results of the *in vitro* assays. Absolute figures and percentages for positivity in the P-gp expression assay (MRK16) and functional efflux assay (calcein-AM).

	AML n=55	**CLL** n= 75	**MM** n= 17
P-gp+ MRK16	**21** 38.2%	**46** 61.3%	**7** 41.2%
Efflux+ Calcein	**27** 49.1%	**42** 56.0%	**7** 41.2%
+ / +	**16** 29.1%	**31** 41.3%	**4** 23.5%

Figure 4.2A shows the results of the calcein-AM accumulation functional assay stratified by P-gp expression (assessed from the MRK16 assay), along with the results of Mann-Whitney tests implemented to determine the level of significant difference between the groups (denoted by a P number, with <0.05 being considered significant). Also shown in Figure 4.2A is a column representing all 55 AML calcein-AM results to illustrate the spread of the data. The numbers at the top of each column shows the median value for those data, and this is also demonstrated by the short horizontal bars bisecting each column.

From these data in Figure 4.2A it can be seen that the P-gp positive group (P-gp+) has a higher median MAF than the P-gp negative group (P-gp-) (19.79 and 3.965 respectively) and that the Mann-Whitney test performed between these data sets show that this difference is highly significant ($P<0.0001$).

Figure 4.2B shows the results of the MRK16 P-gp expression assay stratified by P-gp efflux function (MAF). The medians are shown both on the graphs as horizontal bars and are given numerically above each column. Statistical significance between these groups derived from a Mann-Whitney test is also given.

It can be seen in these data that the efflux positive group (MAF+) has a substantially higher median P-gp expression than the efflux negative group (MAF-) (1.22 and 0.96 respectively), and that this difference is highly statistically significant ($P<0.0001$) .

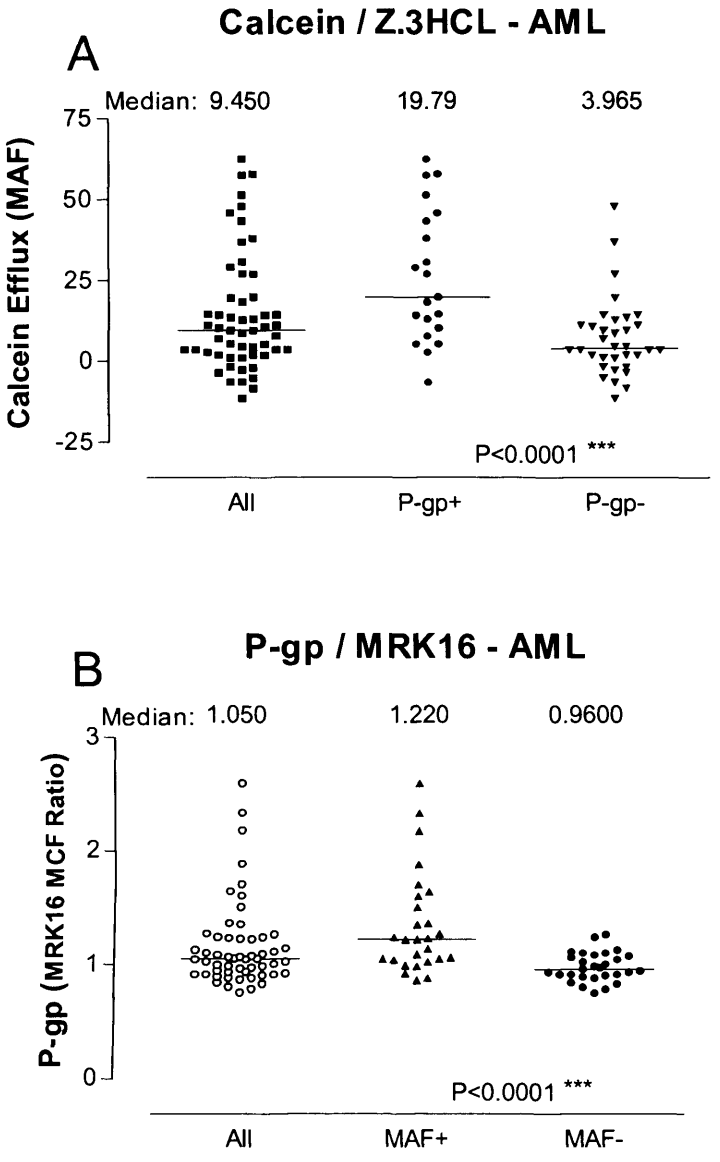


Figure 4.2 AML Calcein-AM accumulation and P-gp expression data. Graph A shows the different levels of calcein accumulation as measured by MAF in relation to different categories of P-gp expression. Graph B shows the P-gp expression as measured by MRK16 in relation to the different categories of calcein accumulation as measured by MAF. The P number denotes the significance of a Mann-Whitney test between the P-gp+ and P-gp- groups in graph A, and MAF+ and MAF- groups in graph B.

Figure 4.3 illustrates the P-gp expression and function profiles of patients samples grouped according to whether they were *de novo* presentation samples (P), samples from patients that had undergone treatment (T), or from patients with secondary or relapsed AML (R). The left-hand three scatter plots show the MAF data from calcein assays, the right three illustrate the data from MRK16 assays, and the bars represent the median of the data.

It can be seen that for the calcein data, there is no statistical difference in P-gp function shown between the three groups, although the median MAF for the treated patients is higher than that of presentation and relapse patient samples (12.62 v 9.45 & 7.29, respectively).

The P-gp expression data shows that samples from treated patients have a significantly higher level of P-gp than the presentation samples (1.23 v 1.04, P= 0.0378). There was no significant difference between either of these two groups and the relapsed group (1.05).

Figure 4.4 shows P-gp expression and function in patient samples grouped according to outcome: complete haematological remission (CR), no remission (NR) and progressive disease (PD). Neither the calcein data nor the MRK16 data show any significant difference in expression or function between the outcome groups.

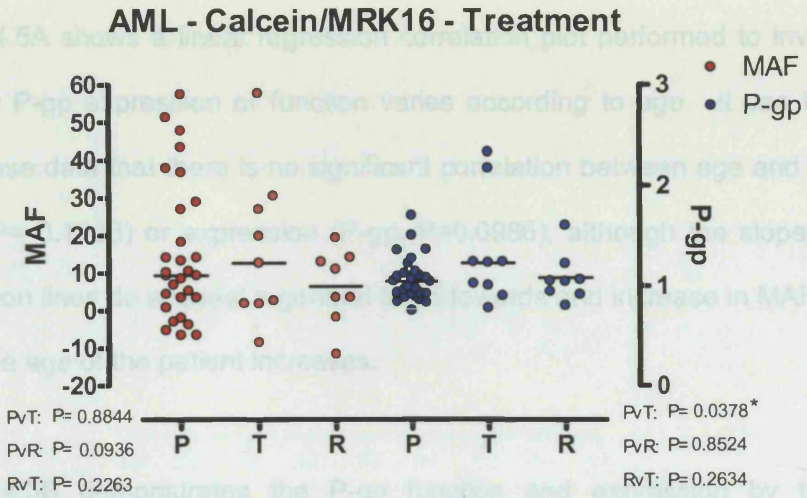


Figure 4.3 AML P-gp expression and function grouped according to treatment. P= presentation sample; T= post treatment sample; R= post relapse sample. The P values represent statistical test performed between the groups.

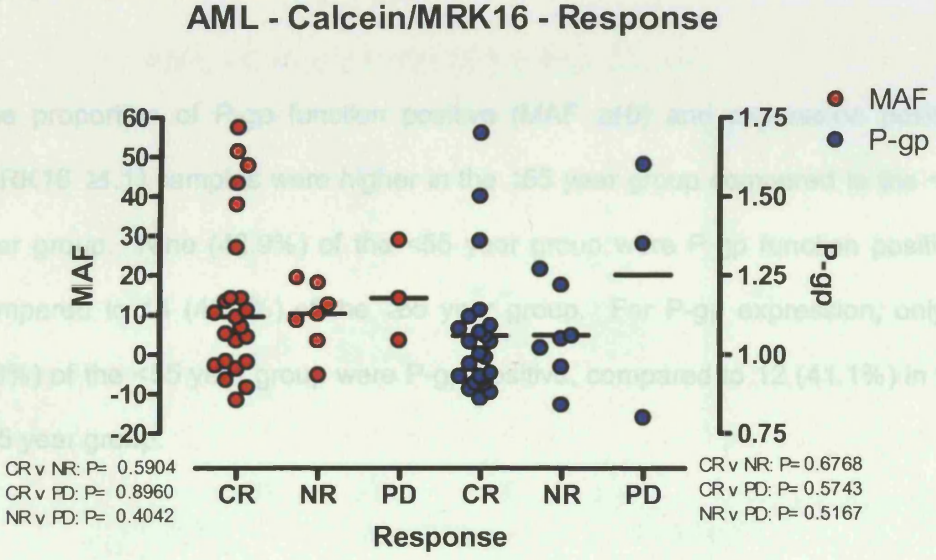


Figure 4.4 AML P-gp expression and function grouped according to patient response. CR= complete remission; NR= no response; PD= progressive disease.

Figure 4.5A shows a linear regression correlation plot performed to investigate whether P-gp expression or function varies according to age. It can be seen from these data that there is no significant correlation between age and function (MAF, P= 0.1933) or expression (P-gp, P=0.0986), although the slopes of the regression lines do suggest a general trend towards and increase in MAF and P-gp as the age of the patient increases.

Figure 4.5B demonstrates the P-gp function and expression by two age groupings: <55 and ≥55 years. Compared to the <55 year group, the median P-gp function was shown to be increased in the ≥55 year group, but this was not statistically significant (4.40 to 9.45, P=0.0988). The median P-gp expression was also higher in the ≥55 year group, and this increase was statistically significant (0.99 to 1.05, P=0.0353).

The proportion of P-gp function positive (MAF ≥10) and expression positive (MRK16 ≥1.1) samples were higher in the ≥55 year group compared to the <55 year group. Nine (42.9%) of the <55 year group were P-gp function positive, compared to 14 (48.3%) of the ≥55 year group. For P-gp expression, only 4 (19%) of the <55 year group were P-gp positive, compared to 12 (41.1%) in the ≥55 year group.

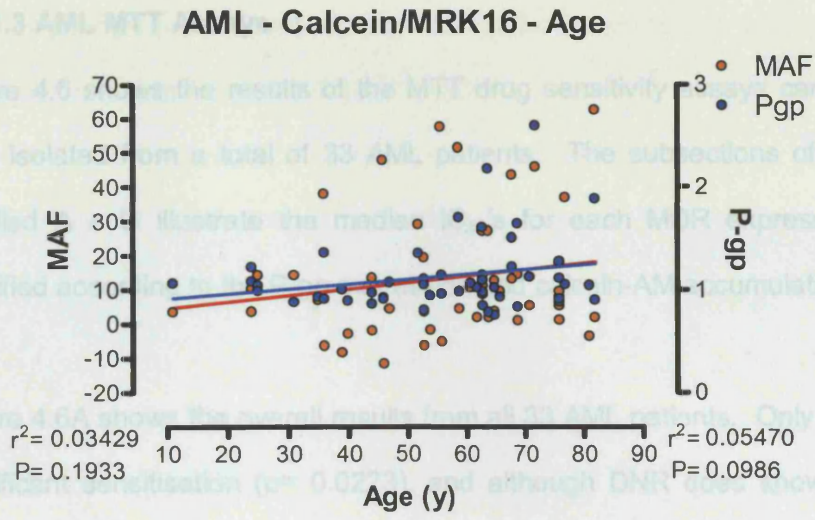


Figure 4.5A AML P-gp expression and function correlated against age. P-gp functional (MAF) and expression (P-gp) data of patient samples correlated using linear regression against patient age.

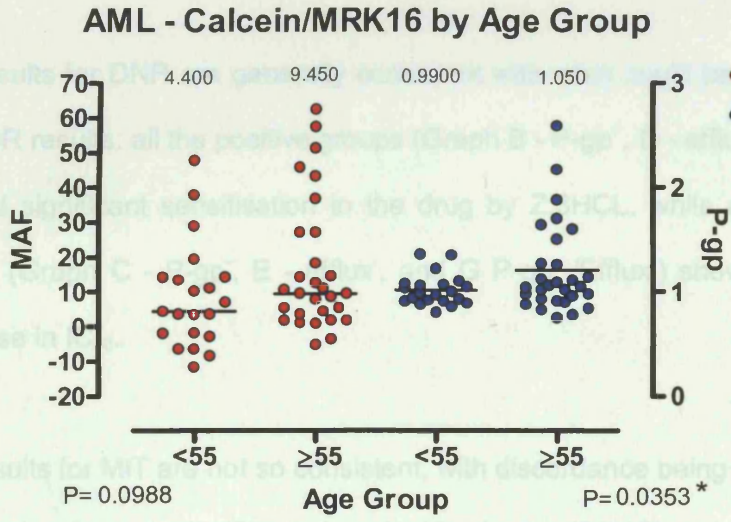


Figure 4.5B AML P-gp expression and function measured against age group. P-gp functional (MAF) and expression (P-gp) data of patient samples in two age groups. The significance of a statistical analysis is denoted by a P value.

4.2.1.3 AML MTT Assays

Figure 4.6 shows the results of the MTT drug sensitivity assays carried out on cells isolated from a total of 33 AML patients. The subsections of the figure, labelled A – G illustrate the median IC_{50}'s for each MDR expression group, stratified according to the P-gp expression and calcein-AM accumulation assays.

Figure 4.6A shows the overall results from all 33 AML patients. Only MIT shows significant sensitisation (p= 0.0223), and although DNR does show a drop in IC_{50}, it is not significant (0.0960). ARA-C is not expected to show significant sensitisation; however it quite consistently (in graphs A, B, C, D, and F) shows that the cells incubated in the presence of ARA-C and Z.3HCL to have a higher IC_{50} than those with ARA-C alone, though this difference did not approach significance.

The results for DNR are generally consistent with what could be predicted from the MDR results: all the positive groups (Graph B - $P\text{-}gp^+$, D - $efflux^+$, and F - +/+) showed significant sensitisation to the drug by Z.3HCL, while all the negative groups (Graph C - $P\text{-}gp^-$, E - $efflux^-$, and G $P\text{-}gp^-$ /$Efflux^-$) show no significant decrease in IC_{50}.

The results for MIT are not so consistent, with discordance being shown in group C ($P\text{-}gp^-$), where a significant drop in IC_{50} in the Z.3HCL cohort is observed (p=0.0340). The remaining negative groups show no significance (graphs E and G). In groups B ($P\text{-}gp^+$) and F ($P\text{-}gp^+$/$Efflux^+$) the expected significant sensitisation is not observed (in contrast to the DNR), and only in group D

(efflux^{+}) do we observe concordance with the DNR results with a significant drop in IC_{50} in the presence of Z.3HCL ($p= 0.0088$).

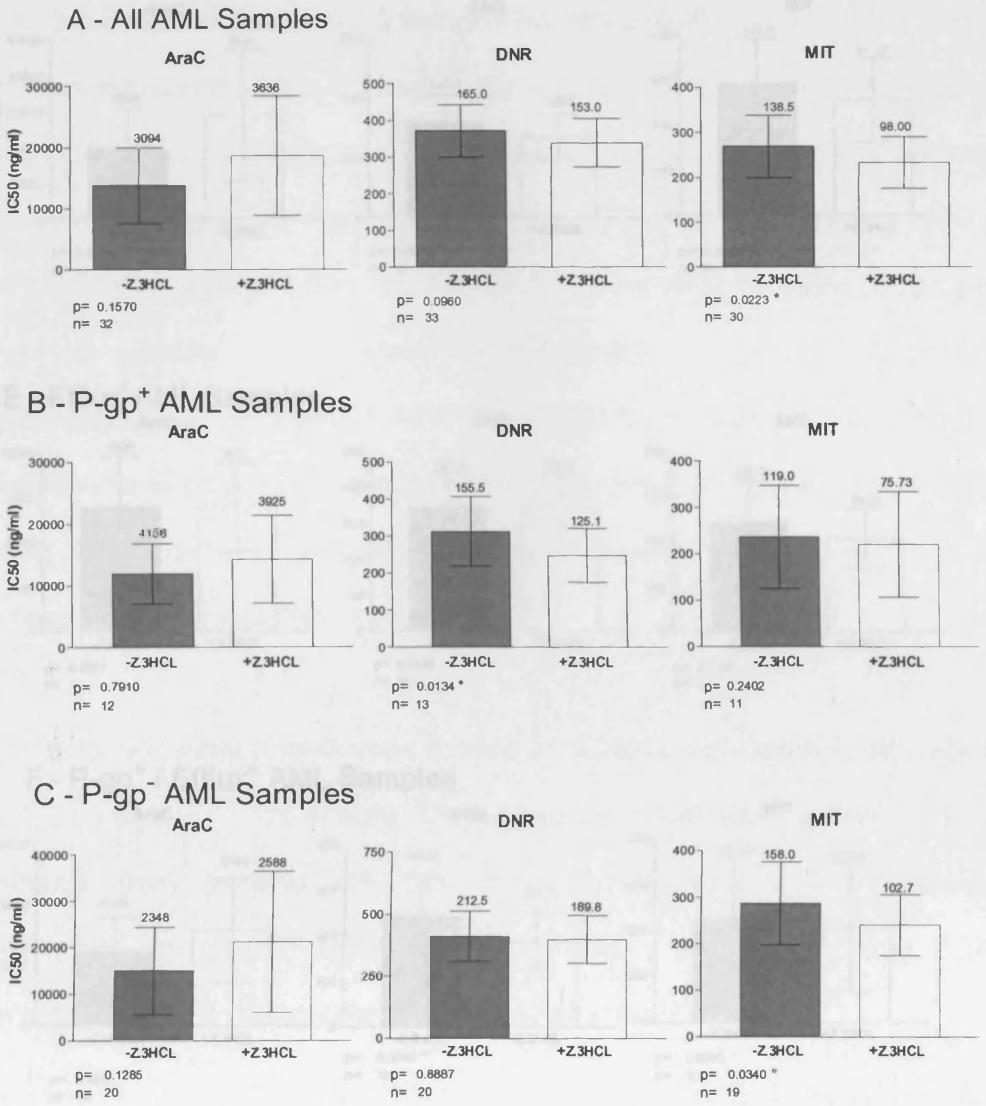
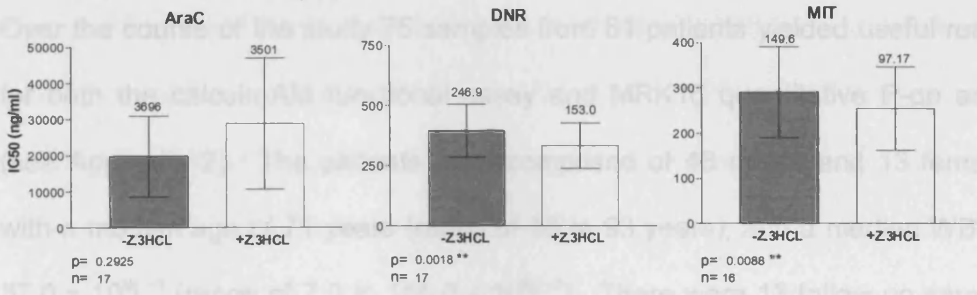
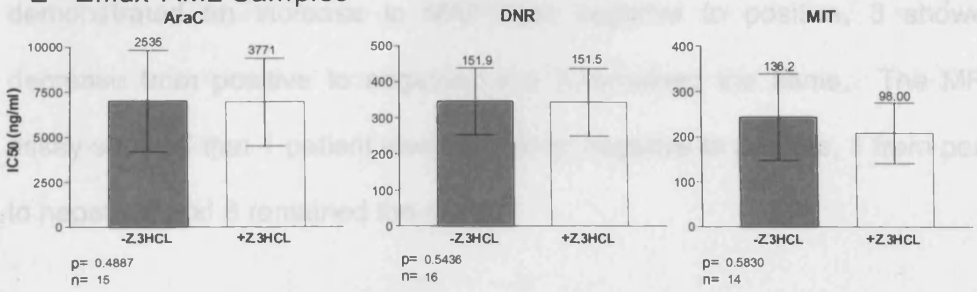


Figure 4.6 AML MTT data. IC50 data for the three drugs, ARA-C, DNR, and MIT, for different MDR expression stratification groups (Graphs A – G), in the presence and absence of Z.3HCL (continued on next page).

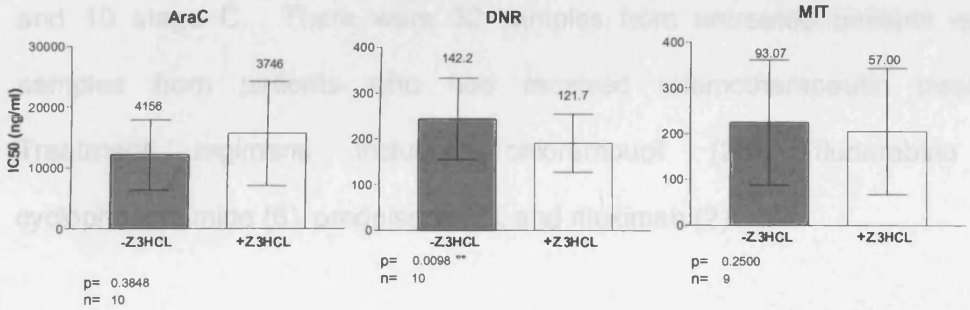
D - Efflux^{+} AML Samples



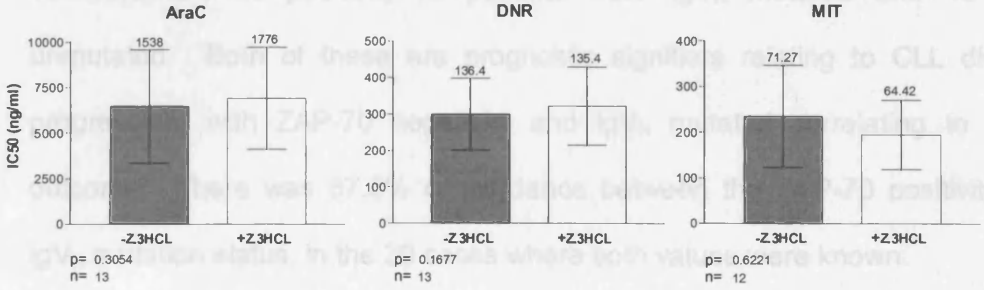
E - Efflux^{-} AML Samples



F - P-gp^{+} / Efflux^{+} AML Samples



G - P-gp^{-} / Efflux^{-} AML Samples



4.2.2 CLL Patient Samples

Over the course of the study 75 samples from 61 patients yielded useful results for both the calcein-AM functional assay and MRK16 quantitative P-gp assay (see Appendix 2). The patients were comprised of 48 males and 13 females, with a median age of 71 years (range of 53 to 93 years), and a median WBC of 37.0 x 10^9L^{-1} (range of 7.0 to 165.0 x 10^9L^{-1}). There were 13 follow up samples from 10 patients, and of these calcein assays showed that 2 patients demonstrated an increase in MAF from negative to positive, 3 showed a decrease from positive to negative, and 5 remained the same. The MRK16 assay showed that 1 patient went from P-gp negative to positive, 1 from positive to negative, and 8 remained the same.

Where known, Binet classification showed 31 patients were stage A, 18 stage B, and 10 stage C. There were 32 samples from untreated patients and 43 samples from patients who had received chemotherapeutic treatment. Treatment regimens included chlorambucil (26), fludarabine (7), cyclophosphamide (6), prednisone (2), and rituximab (2).

Where results were available, 31 patients were found to be ZAP-70 negative and 10 were ZAP-70 positive; 19 patients were IgV_H mutated and 13 were unmutated. Both of these are prognostic signifiers relating to CLL disease progression, with ZAP-70 negativity and IgV_H mutated correlating to better outcome. There was 87.5% concordance between the ZAP-70 positivity and IgV_H mutation status, in the 30 cases where both values were known.

4.2.2.1 CLL MDR Assay Correlation

Figure 4.7 shows the correlation between P-gp expression and MDR efflux function for Z.3HCL (Figure 4.7A) and VPM (Figure 4.7B) as the P-gp inhibitor. As with the AML patients, this allows for a comparison between the two inhibitors in the *in vitro* CLL environment.

It can be seen that for both Z.3HCL and VPM calcein-AM assays the correlation with P-gp expression was highly significant ($P<0.0001$ and 0.0003 respectively), but due to considerable scatter the r^2 is extremely low (0.2549 for Z.3HCL and 0.1702 for VPM).

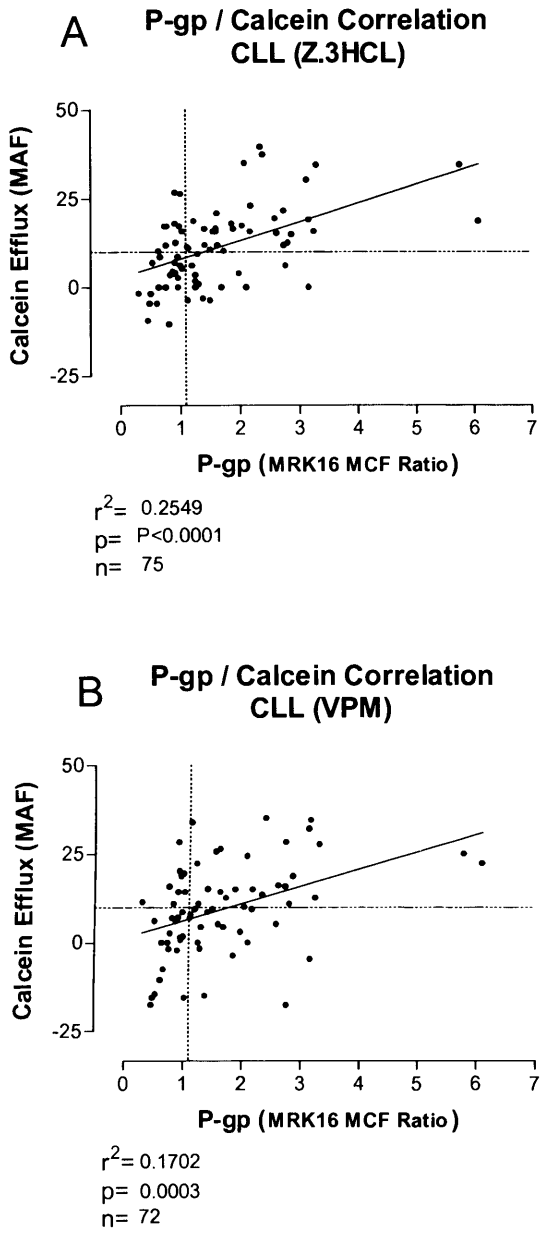


Figure 4.7 P-gp expression and calcein-AM accumulation correlation in CLL. Graphs showing the correlation between P-gp expression (MRK16) and functional Calcein dye efflux inhibition (MAF). A p-value is shown to indicate significance.

4.2.2.2 CLL MDR Assays

Figure 4.8A illustrates the results of the calcein-AM functional assay, stratified by P-gp expression (assessed from the MRK16 assay), and also shows the results of Mann-Whitney tests performed in order to determine any significant difference between the groups. Also shown in Figure 4.8A is a column representing all 75 CLL calcein-AM results to show the spread of the data. The numbers at the head of each column show the median value for those data, and this is also illustrated by the short horizontal bars bisecting each column.

From these data it can be seen that there is a significant difference in median MAF between the P-gp+ and the P-gp- groups, with the P-gp+ group having a higher median MAF than the P-gp- group (15.52 and 6.10 respectively, P=0.0024).

Figure 4.8B illustrates the results of the MRK16 P-gp expression assay stratified by P-gp efflux function (MAF). The medians are shown on the graphs as horizontal bars and are given numerically above each column. Statistical significance between these groups derived from Mann-Whitney tests is also shown.

It can be seen that in these data that the efflux positive group (MAF+) has a higher median level of P-gp expression than the MAF negative group (MAF-) (1.68 and 0.97 respectively) and that this difference is statistically significant ($p<0.0001$).

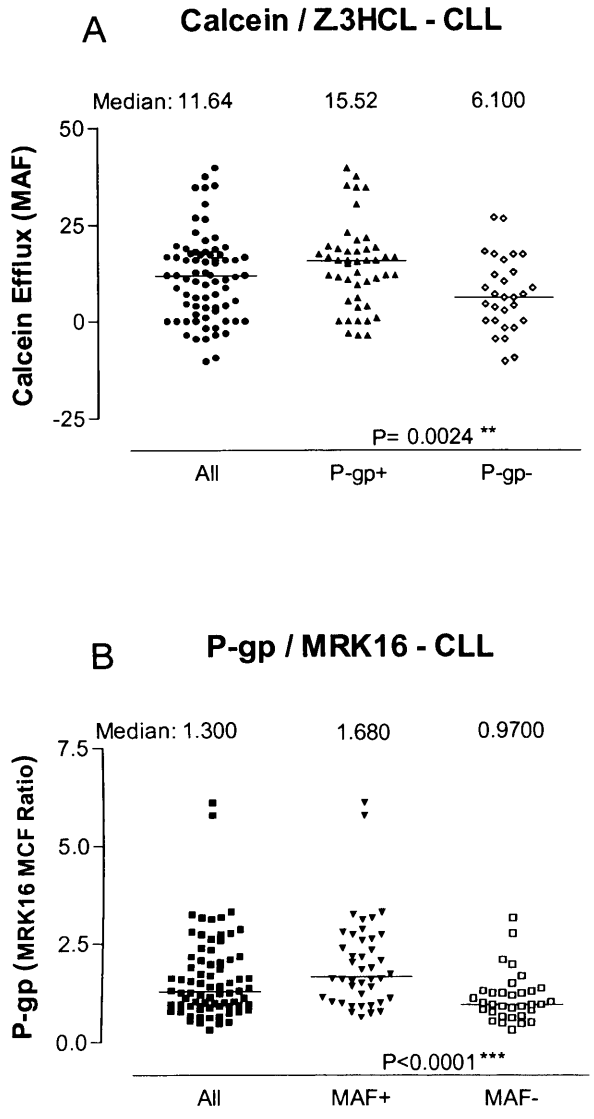


Figure 4.8 CLL Calcein assay and P-gp expression assay data. Graph A shows the different levels of calcein efflux (MAF) in relation to different categories of P-gp expression. Graph B shows the P-gp expression (MRK16) in relation to the different categories of calcein accumulation as measured by MAF. The P number denotes the significance of a Mann-Whitney test between the P-gp+ and P-gp- groups in graph A, and MAF+ and MAF- groups in graph B.

Figure 4.9 shows the median P-gp expression and function for patient samples grouped by Binet stage. It can be seen from the data that there is no significant difference either in function (MAF) nor in expression (P-gp) between any of the three groups. The P values indicated represent statistical test performed between the three stages.

Figure 4.10 demonstrates the median P-gp expression and function between samples from patients who either received treatment (T) or were untreated (U). It can be seen that the median MAF was slightly lower in the treated group than in the untreated group, but not to the level of statistical significance (10.69 v 13.79, P=0.6753). The P-gp expression of the treated group was also lower than the untreated group, but with the difference approaching significance (1.58 v 1.01, P=0.0545).

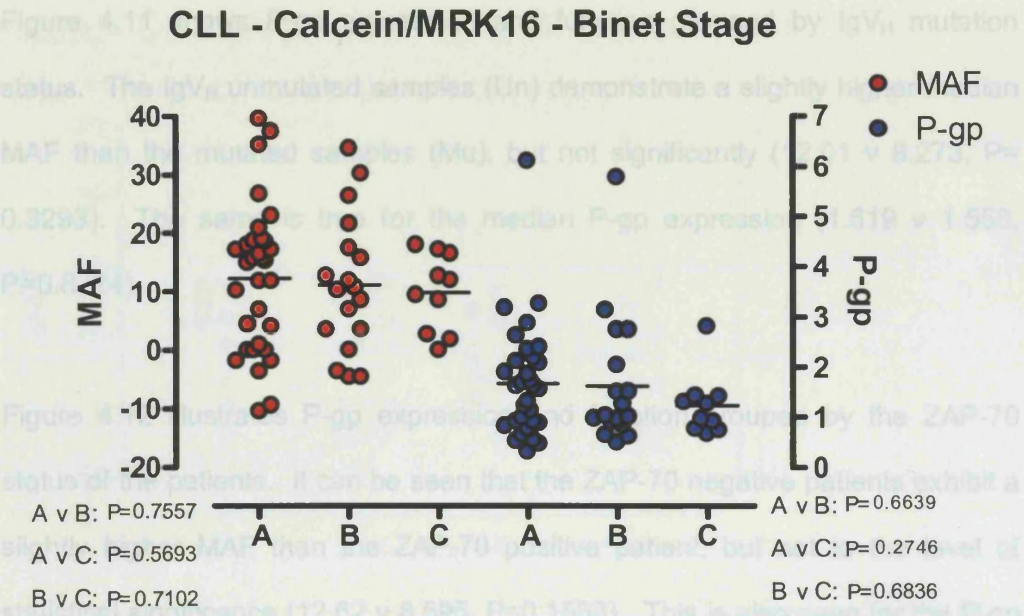


Figure 4.9 CLL P-gp expression and function grouped by Binet Stage. A= Binet stage A; B= Binet stage B; C= Binet stage C.

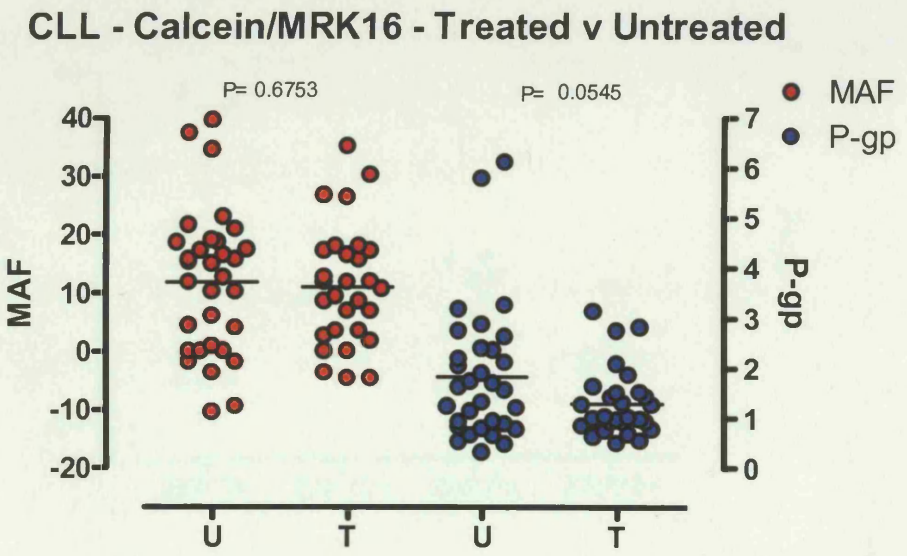


Figure 4.10 CLL P-gp expression and function grouped by treatment status. U= untreated, T= treated.

Figure 4.11 shows P-gp expression and function grouped by IgV_H mutation status. The IgV_H unmutated samples (Un) demonstrate a slightly higher median MAF than the mutated samples (Mu), but not significantly (12.01 v 8.273, P= 0.3293). The same is true for the median P-gp expression (1.619 v 1.558, P=0.8724).

Figure 4.12 illustrates P-gp expression and function grouped by the ZAP-70 status of the patients. It can be seen that the ZAP-70 negative patients exhibit a slightly higher MAF than the ZAP-70 positive patient, but not to the level of statistical significance (12.62 v 8.595, P=0.1563). This is also seen for the P-gp expression data (1.709 v 1.278, P=0.3575).

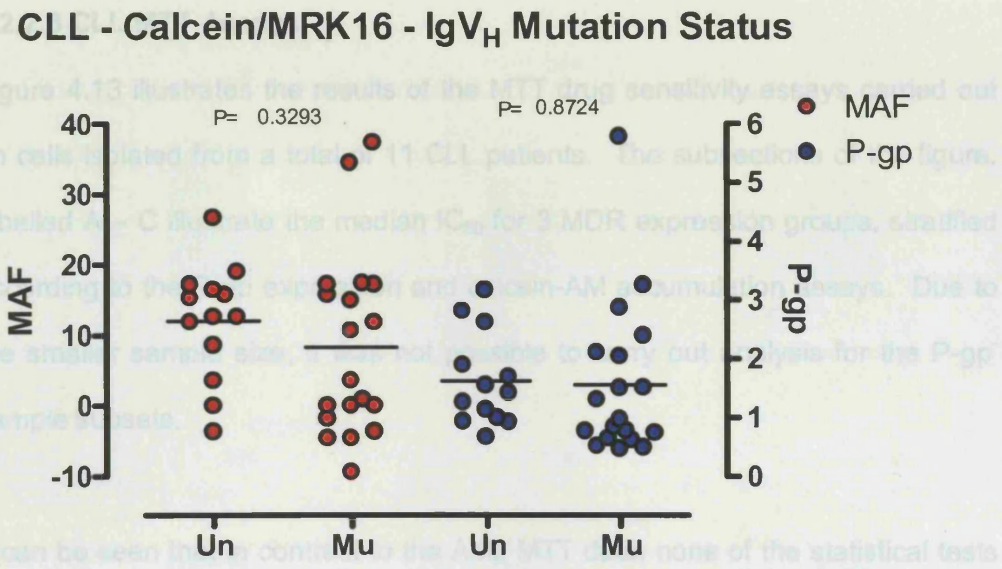


Figure 4.11 CLL P-gp expression and function grouped by IgV$_H$ mutation.

Un= mutated, Mu= mutated.

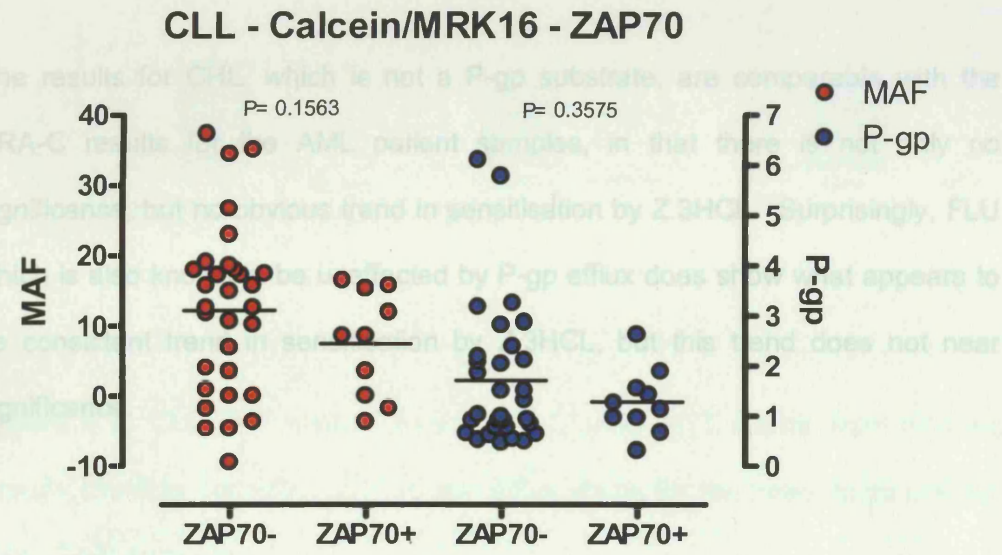


Figure 4.12 CLL P-gp expression and function grouped by ZAP-70 expression.

4.2.2.3 CLL MTT Assays

Figure 4.13 illustrates the results of the MTT drug sensitivity assays carried out on cells isolated from a total of 11 CLL patients. The subsections of the figure, labelled A – C illustrate the median IC_{50} for 3 MDR expression groups, stratified according to the P-gp expression and calcein-AM accumulation assays. Due to the smaller sample size, it was not possible to carry out analysis for the $P\text{-}gp^-$ sample subsets.

It can be seen that in contrast to the AML MTT data, none of the statistical tests performed between the data sets yielded a significant difference, even though a clear trend in IC50 is demonstrated by the addition of Z.3HCL for the P-gp substrate DNR. This lack of significance may be explained by the relatively small number of patient samples analysed.

The results for CHL, which is not a P-gp substrate, are comparable with the ARA-C results for the AML patient samples, in that there is not only no significance, but no obvious trend in sensitisation by Z.3HCL. Surprisingly, FLU which is also known to be unaffected by P-gp efflux does show what appears to be consistent trend in sensitisation by Z.3HCL, but this trend does not near significance.

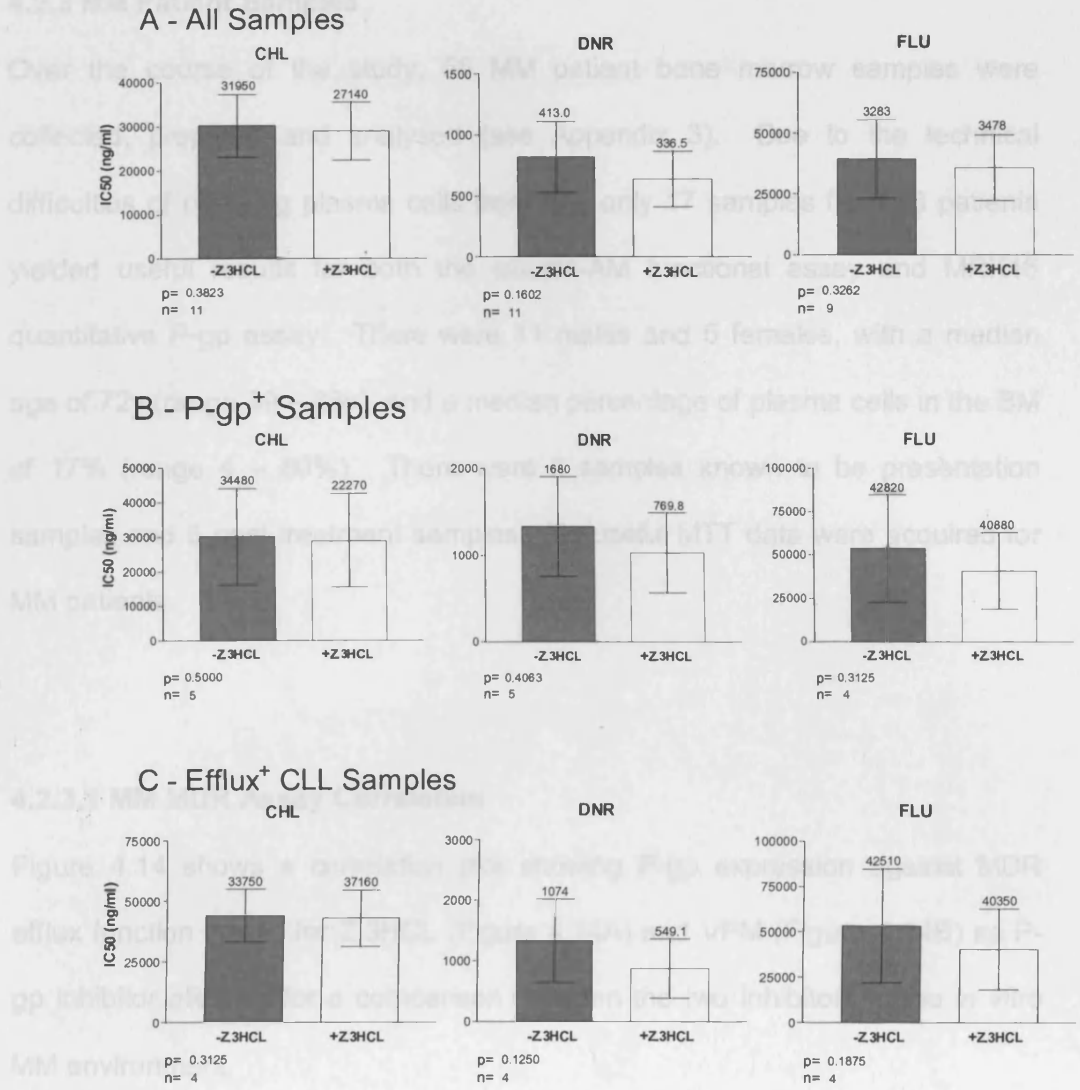


Figure 4.13 CLL MTT data. Graphs A – C show MTT results from different groups stratified according to P-gp and efflux status for the three drugs utilized, CHL, DNR and FLU.

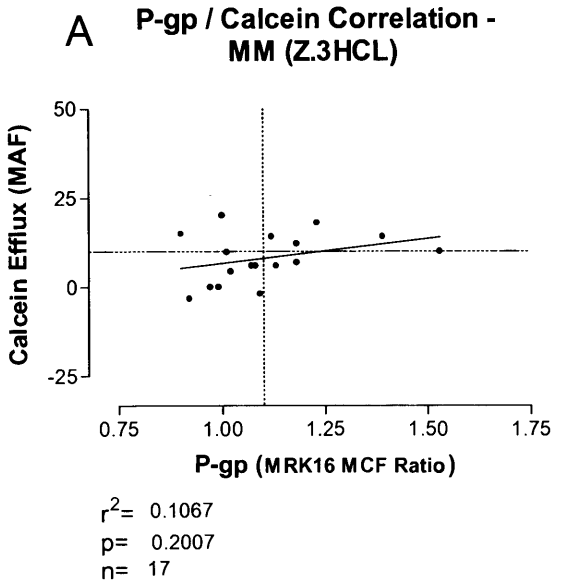
4.2.3 MM Patient Samples

Over the course of the study, 56 MM patient bone marrow samples were collected, prepared and analysed (see Appendix 3). Due to the technical difficulties of purifying plasma cells from BM, only 17 samples from 16 patients yielded useful results for both the calcein-AM functional assay and MRK16 quantitative P-gp assay. There were 11 males and 5 females, with a median age of 72y (range 49 – 88y), and a median percentage of plasma cells in the BM of 17% (range 4 – 80%). There were 9 samples known to be presentation samples and 6 post treatment samples. No useful MTT data were acquired for MM patients.

4.2.3.1 MM MDR Assay Correlation

Figure 4.14 shows a correlation plot showing P-gp expression against MDR efflux function (MAF) for Z.3HCL (Figure 4.14A) and VPM (Figure 4.14B) as P-gp inhibitor allowing for a comparison between the two inhibitors in the *in vitro* MM environment.

It can be seen that for both the Z.3HCL and VPM calcein-AM assays the correlations with P-gp expression were very weak, with no statistical significance (P=0.2007 and 0.0939 respectively). The r^2 values denoting goodness of fit are also extremely low (0.1067 for Z.3HCL and 0.2161 for VPM). Overall, the assay results from the MM cohort of patient samples appeared to be even more heterogeneous than for the AML and CLL samples. This, coupled with the relatively low sample number (n=17 for Z.3HCL, and n= 14 for VPM), may help explain the failure to find statistically meaningful correlation.



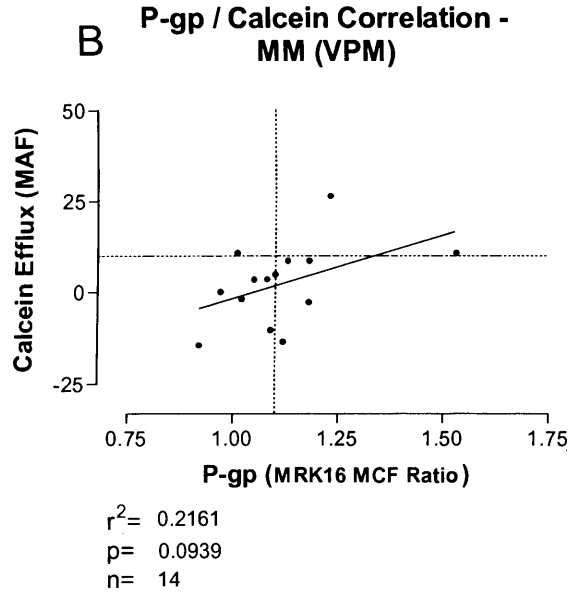


Figure 4.14 P-gp expression and calcein accumulation correlation in MM. These graphs show the correlation between P-gp expression measured by MRK16 MoAb and functional Calcein dye efflux inhibition, as measured by MAF. A P-value is shown to indicate significance.

4.2.3.2 MM MDR Assays

Figure 4.15A illustrates the results of the calcein-AM functional assay, stratified by P-gp expression (assessed from the MRK16 assay). Also shown in Figure 4.15A is a column representing all 17 MM calcein-AM results to illustrate the spread of the data. The numbers at the head of each column show the median value for those data, and this is also illustrated by the short horizontal bars bisecting each column.

From these data in Figure 4.15A it can be seen that the P-gp positive group (P-gp+) exhibits a higher median MAF than the P-gp negative group (P-gp-) (12.22 and 5.250 respectively), and that this difference is statistically significant (P=0.0351).

Figure 4.15B illustrates the results of the MRK16 P-gp expression assay stratified by P-gp efflux function (MAF). The medians are shown both on the graphs as horizontal bars and are given numerically above each column.

It can be seen that in these data that although the efflux positive group (MAF+) has a higher median P-gp expression than the efflux negative group (MAF-) (1.18 and 1.045, respectively), this difference was not found to be statistically significant (P= 0.0665).

This finding is discordant with the results obtained from the AML and CLL studies; however, a caveat may be made regarding the relatively low sample size for this result and it may be reasonable to assume that, since this result

nears significance, that if more data were to be accumulated the observed difference in medians would become significant.

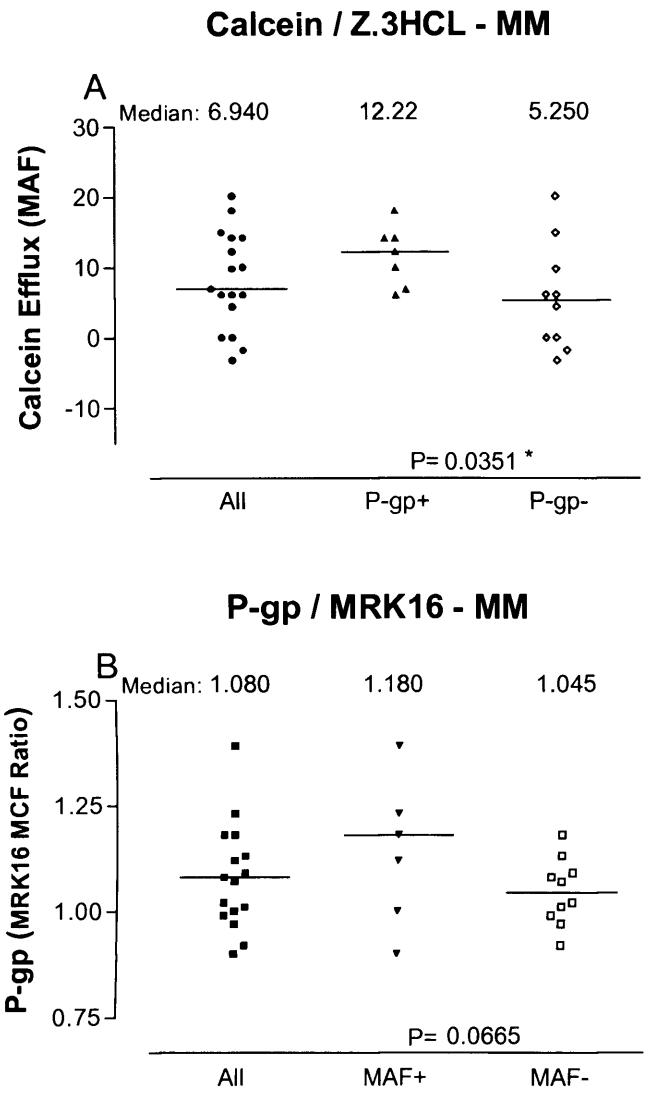


Figure 4.15 MM Calcein assay and P-gp expression assay data. Graph A shows the different levels of calcein accumulation as measured by MAF in relation to different categories of P-gp expression. Graph B shows the P-gp expression as measured by MRK16 in relation to the different categories of calcein accumulation as measured by MAF. The P number denotes the significance of a Mann-Whitney test between the P-gp+ and P-gp- groups in graph A, and MAF+ and MAF- groups in graph B.

Figure 4.16 shows the median P-gp expression and function data for patient samples grouped according to treatment status. Presentation samples (P) and samples from patients who had undergone chemotherapy (T) were compared statistically. It can be seen that there was no significant difference between the two groups for function (MAF) (9.830 v 8.065, P=0.2804) or for expression (P-gp) (1.020 v 1.135, P=0.2721).

Figure 4.17 illustrates P-gp expression and function correlated against the percentage of plasma cells (PC) in the patient BM samples. The results of the linear regression show quite clearly that there is no relationship between the number of PC's and MAF (r^2= 0.0045, P=0.8277) or P-gp (r^2=0.090, P=0.3201).

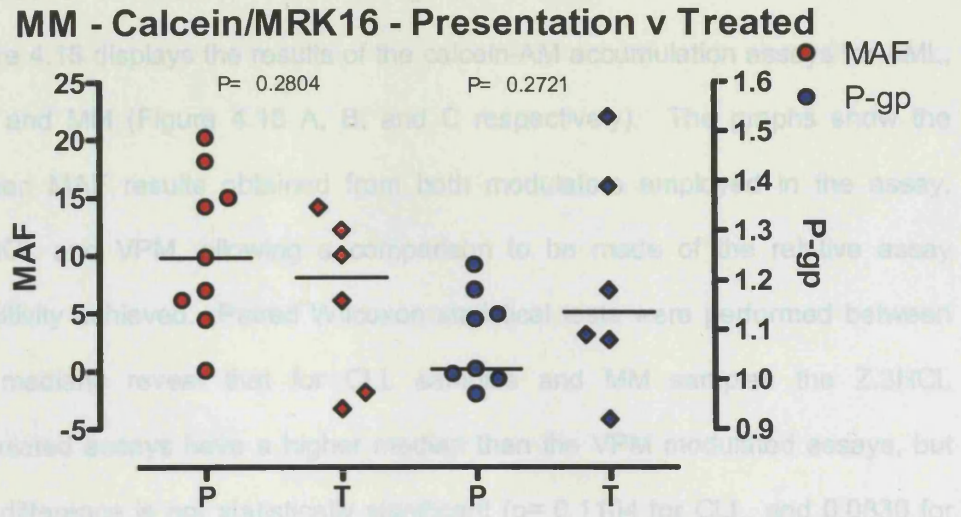


Figure 4.16 MM P-gp expression and function grouped by treatment.. P= presentation, T= treated.

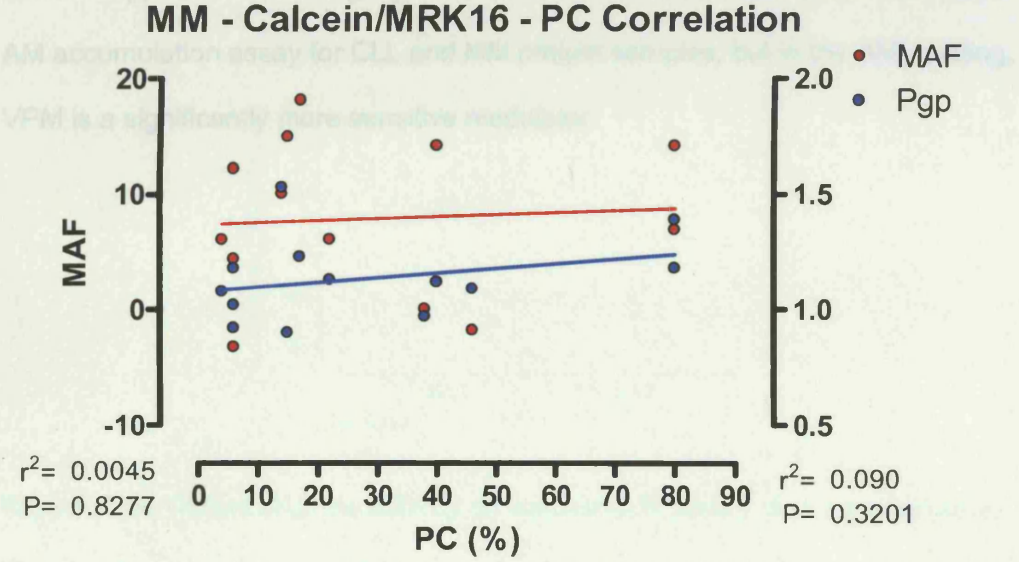


Figure 4.17 MM P-gp expression and function correlated against BM plasma cells. Linear regression displaying correlation against percentage PC in BM.

4.2.4 Level of Calcein-AM Sensitivity

Figure 4.18 displays the results of the calcein-AM accumulation assays for AML, CLL and MM (Figure 4.18 A, B, and C respectively). The graphs show the median MAF results obtained from both modulators employed in the assay, Z.3HCL and VPM, allowing a comparison to be made of the relative assay sensitivity achieved. Paired Wilcoxon statistical tests were performed between the medians reveal that for CLL samples and MM samples the Z.3HCL modulated assays have a higher median than the VPM modulated assays, but that difference is not statistically significant ($p= 0.1104$ for CLL, and 0.0830 for MM). The results for the AML however show the converse, the VPM modulated assays have a higher median MAF than the Z.3HCL modulated assays, and that difference is statistically significant ($p= 0.0001$).

Z.3HCL appears to be a slightly more sensitive modulator for use in the calcein-AM accumulation assay for CLL and MM patient samples, but in the AML setting, VPM is a significantly more sensitive modulator.

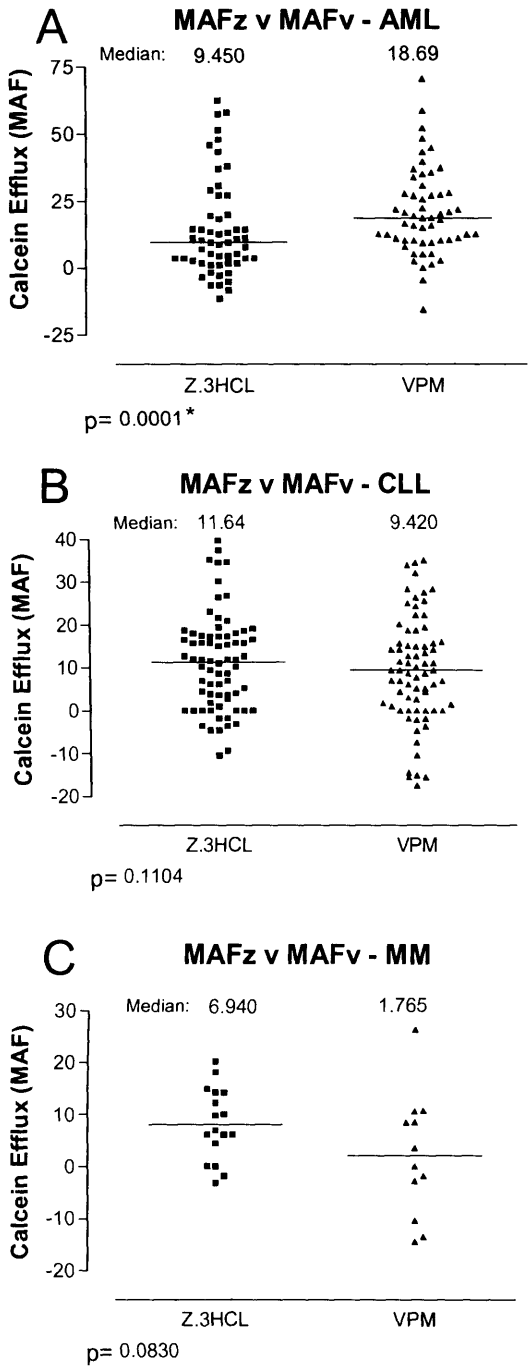


Figure 4.18 Differential sensitivity in calcein-AM assay due to modulator. The results of the calcein-AM assay for each tumour type (A - AML; B - CLL; C – MM) showing the median MAF obtained from the two P-gp modulators utilised, Z.3HCL and VPM.

4.2.5 Tests and Statistics

Not all AML, CLL, and MM MDR results were found to be normally distributed, so non-parametric Mann-Whitney or Wilcoxon paired tests were used, and since the expected outcome could be predicted (i.e., P-gp$^+$ cells to have a higher MAF than P-gp$^-$ cells), 1-tail unpaired t-tests were performed where appropriate. Where t-test were employed, an F-test to test for unequal variances was also performed and where the variances between the two data groups were found to be significant, Welch's Correction was implemented to compensate.

4.3 Discussion

In AML, MDR induced by P-gp over-expression has been shown to be a major prognostic signifier of adverse outcome and treatment failure[327-329]. There is a real case for the targeted use of P-gp modulators in those patients for which P-gp over-expression has been identified, provided that the active compound is specific and has a low toxicity profile. Even when the inherent toxicity has an adverse effect overall, it has been shown that for those individuals with high P-gp expression, the benefits of modulation may well outweigh the risks[267,282].

It is doubtful if P-gp expression plays any meaningful role in drug efflux in CLL, mainly due to the use of non-P-gp transported drugs such as chlorambucil and fludarabine in its treatment, and it remains to be seen if the non-efflux mechanisms attributed to P-gp could exert some influence[330]. The situation for MM is not clear and studies have not shown a clear cut prognostic significance, but since P-gp substrates such as daunorubicin and vincristine are utilised in the treatment of MM, there may yet be a case for P-gp modulation in the treatment of this disease[200,284].

The data presented in this chapter show that Z.3HCL is a valuable component of two of the three *in vitro* assays employed to investigate P-gp mediated MDR in clinical samples. In order to use P-gp modulating compounds such as Z.3HCL as part of a targeted therapeutic regimen, a rapid and accurate assessment of the patient's P-gp status needs to be implemented. Moreover, by combining P-gp expression with functional efflux data a more precise evaluation of the patients MDR risk can be made. Since both these tests are performed by flow cytometry, the evaluation can be done relatively rapidly.

In the cohort of 48 AML patients there was evidence from the presentation and post-treatment samples to support the hypothesis that P-gp expression is higher following chemotherapeutic treatment, as this cytotoxic insult should trigger P-gp upregulation. P-gp was found to be significantly higher in the post-treatment group, and went from a median figure considered negative for P-gp over-expression (1.04) to one considered positive (1.23). It must also be noted that because of the lack of an extra marker for efficient blast gating on the FACS (such as anti CD45), the data from post-treatment samples from patients in CR are likely to have included a high proportion of normal BM mononuclear cells. It is likely that the MDR profile of these BM cells may well have been divergent from these patients' leukaemic blasts, and this represents a potential confounding factor when considering these particular results.

The functional calcein assay showed a similar increase in the post-treatment group, and although this was not shown to be statistically significant, here too the median MAF went from negative (9.45) to positive (12.62). This finding was not seen in the equivalent sample groups from CLL and MM patients, which, in the case of the CLL patients reinforces the idea that P-gp is not a significant factor in the generation of MDR in this disease. It is likely the very low number of MM samples eligible for analysis precludes the ability to draw meaningful conclusions about the existence of this phenomenon in MM.

Samples from patients with secondary or relapsed AML did not have any significant difference in P-gp levels to the presentation sample group. The caveat to these data is that the post treatment group was represented by a relatively small number of samples (n=9).

Across the three disease types, there was no other influencing factor that significantly altered the expression or function of P-gp that could be measured by the calcein and MRK16 assays: IgV_H, ZAP-70 and Binet status in CLL, or the percentage of plasma cells in MM. Other studies have stated a correlation between age and P-gp function that was not found to be of significance in this study. Although it can be seen from Figure 4.5A however, that in AML there is a trend for increasing P-gp expression and function as patient age increases, and that the failure to reach significant concordance with the observations of previous studies could possibly be due to factors such as the relatively lower patient numbers involved in this study[198,199]. A greater level of concordance can be seen when the MDR data is split into two age groups of <55 and ≥55 years of age, as is demonstrated in Figure 4.5B. Here we can see that there is a significant increase in the median P-gp expression levels for the ≥55 year age group. It was also observed that the relative proportions of P-gp positivity and function was increased in the ≥55 year age group.

Another factor that must be considered as an inherent weakness in the design of the flow cytometry based AML assays is the omission of co-labelling with a blast-specifying fluorescent antibody, such as anti-CD45. Its use would have increased the confidence with which the tumour cells under examination could have been gated and other high P-gp expressing cells, such as NK cells, could have been excluded. However, it can also be argued that the use of anti-CD45 in itself would not be stringent enough to exclude all potentially confounding cell types. The use of further markers would render the assay unnecessarily complex and could introduce technical errors, such as spectral overlap from improperly calibrated channel compensation. It may be worth considering that a

basic 3-colour FACS is now insufficiently equipped for adequate analysis of MDR, and that any future work should make use of more advanced 4 or even 6-colour FACS analysers.

Another factor to consider when attempting to assess the impact of patient MDR status upon therapeutic response, is what proportion of those patients were actually treated chemotherapeutically with curative intent. Out of 55 samples, 16.4% were post-treatment, but there were only 6 patients (5 *de novo* and 1 relapsed) where both a treatment and post-treatment sample were received. Since only one of these patients was MDR negative this cohort can only give minimal information regarding the impact of P-gp expression and function on outcome.

Drug sensitivity data by use of the MTT assay is slower to produce results but it can be argued that it is potentially a more accurate indication of the MDR status of a patient's cells since it is a direct measurement of both the level of drug efficacy with and without P-gp modulation. It can therefore be reasoned that a combination of these tests should be used to stratify patients into levels of MDR expression. Since some level of discordance between the tests can arise, most probably due to technical limitations, a scoring system based on the number of tests found positive may be employed to give an indication of that patient's likelihood of treatment failure due to the effects of P-gp over-expression and the MDR effect.

5. *In Vitro* Studies – P-glycoprotein and Apoptosis

5.1 Introduction

P-gp has been implicated as an influencing factor of caspase-dependent apoptotic signalling, reducing the sensitivity of P-gp over expressing cells to certain forms of apoptotic stimuli. One of the ways in which P-gp may act on this pathway is by trafficking sphingomyelin to the outer leaflet of the plasma membrane, thus reducing the available cytoplasmic ceramide pool. It is suggested that ceramide, a degradation product of membrane sphingomyelin, can act as a second messenger for drug or radiation induced apoptotic initiation[184]. Other studies have shown that P-gp expression can inhibit caspase 8 activation following death receptor ligation (e.g., FAS), and it is hypothesised that this may be caused by P-gp induced perturbations in the plasma membrane composition local to the action site.

On the cell surface, FAS is partitioned into sphingolipid and cholesterol rich membrane rafts. Upon ligation, FAS aggregates to form clusters in a ceramide-dependant manner. These FAS clusters then associate with an adapter molecule called FADD (Fas-associated death domain protein) and through recruitment of inactive caspase 8 forms the death inducing signal complex (DISC), which activates caspase 8 and initiates the apoptotic cascade. Since P-gp has not been shown to inhibit either the formation of DISC or recruitment of caspase 8 to the DISC apparatus, it is thought that it modulates the apoptotic signal through interfering with the autocatalysis of caspase 8, possibly through the disruption of the sphingolipid and cholesterol rafts[187,331].

Since several studies have suggested that P-gp modulation could reverse the anti-apoptotic effect, the inhibitory effect of P-gp on caspase-dependent apoptosis and the effect of Z.3HCL to ameliorate this phenomenon was investigated. Ionising radiation was primarily used to initiate apoptosis, having been shown by previous studies to be affected by P-gp over-expression[108,332]. The use of radiation, as opposed to a cytotoxic drug, obviated the need to compensate for MDR efflux.

Initially, the study looked at the cleavage of PARP (poly-(ADP ribose) polymerase (p116), a caspase 3 substrate) into the p85 fragment, a process considered to be a hallmark of apoptosis. This was attempted through the use of western blots to ascertain differential apoptotic signals between $P\text{-}gp^-$, $P\text{-}gp^+$ and $P\text{-}gp^+$ modulated cells. Cell death was measured using the MTT assay and morphologically from cytospins. Later in the study, the JC-1 mitochondrial membrane potential flow cytometry assay was employed to try and obtain greater sensitivity. Subsequently the Annexin-V flow cytometry assay was used because of its sensitivity and ease of use.

5.1.1 P116, P85 and other Apoptotic Protein Markers

Western blotting was used in an attempt to ascertain whether apoptotic responses were affected by P-gp expression and if these effects were modulated by Z.3HCL. Chapter 2.2.5 refers to the protocol and rationale.

5.1.2 MTT Cell Viability Assay

An MTT cell viability assay was often used in conjunction with the one or more of the other assays in order to verify the results (see Chapter 2.1.2.3 for details).

5.1.3 JC-1 Assay

JC-1 was used to visualise early apoptotic changes to the mitochondrial membrane potential in response to various apoptotic stimuli and modulation with Z.3HCL (refer to Chapter 2.2.4 for protocol).

5.1.4 Annexin V Assay

The annexin V assay is flow cytometer based and allows the detection of very early apoptotic changes. In many cells, phosphatidylserine (PS) residues display an asymmetric plasma membrane distribution pattern in that they reside only on the inner envelope of the membrane (see Chapter 2.2.6). For the purpose of these experiments it was found that measuring the annexin V +, PI- cells provided the most consistent data (Figure 5.1).

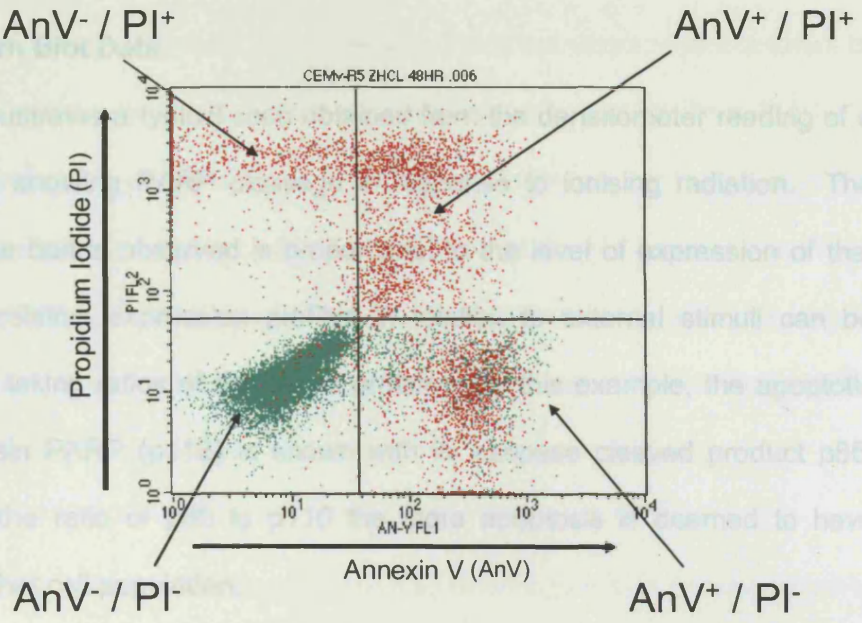


Figure 5.1 Annexin V and PI flow cytometry staining. Showing four quartiles of staining intensity in CEMv cells: live cells (AnV^- / PI^-); apoptotic cells (AnV^+ / PI^-); late stage apoptotic cells (AnV^+ / PI^+); and necrotic cells (AnV^- / PI^+)

5.2 Results

5.2.1 Western Blot Data

Figure 5.2 illustrates a typical scan obtained from the densitometer reading of a western blot showing PARP cleavage in response to ionising radiation. The density of the bands observed is proportional to the level of expression of that protein, so relative expression profiles in relation to external stimuli can be recorded by taking ratios of the target proteins. In this example, the apoptotic marker protein PARP (p116) is shown with its caspase cleaved product p85. The higher the ratio of p85 to p116 the more apoptosis is deemed to have occurred in that cell population.

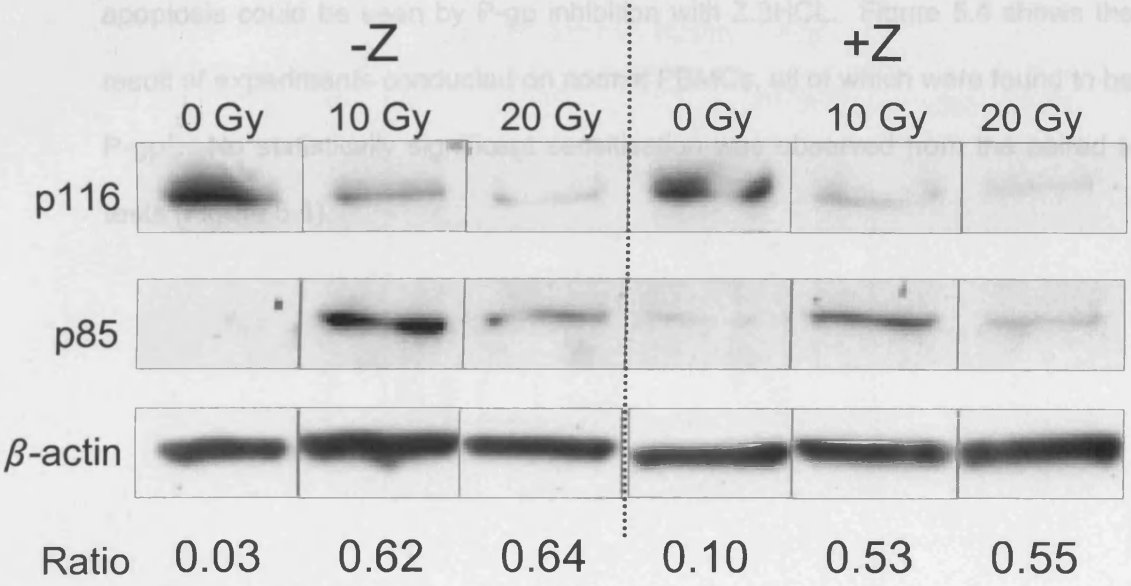


Figure 5.2 Western blot data acquisition after 24hr in CEMv cells. Developed film scanned on densitometer and level of expression determined by band intensity. Amount of apoptosis determined from ratio of the cleavage product p85 against p116. –Z= no Z.3HCL (control); +Z = cells incubated with 100nM Z.3HCL. The housekeeper protein β-actin was used to show any variance in loading volume.

Figure 5.3 shows the differential apoptosis induced in cells isolated from CLL patients by exposure to γ-radiation. The amount of apoptosis was indicated by the ratio of PARP cleavage by effector caspases (p85 / p116). The CLL cells were also tested for MDR markers and all were found to be P-gp$^+$ (data not shown). The CLL cells were exposed to 0, 5, and 10Gy of γ-radiation and then incubated at 37°C with and without 100nM Z.3HCL. Protein lysates were made at 6hr and 24hr. Paired t-tests on the results show that any differences between the –Z and +Z.3HCL cells are not significant.

PBMCs isolated from normal donors were also used to see if any sensitisation to apoptosis could be seen by P-gp inhibition with Z.3HCL. Figure 5.4 shows the result of experiments conducted on normal PBMCs, all of which were found to be P-gp$^+$. No statistically significant sensitisation was observed from the paired t-tests (Figure 5.4).

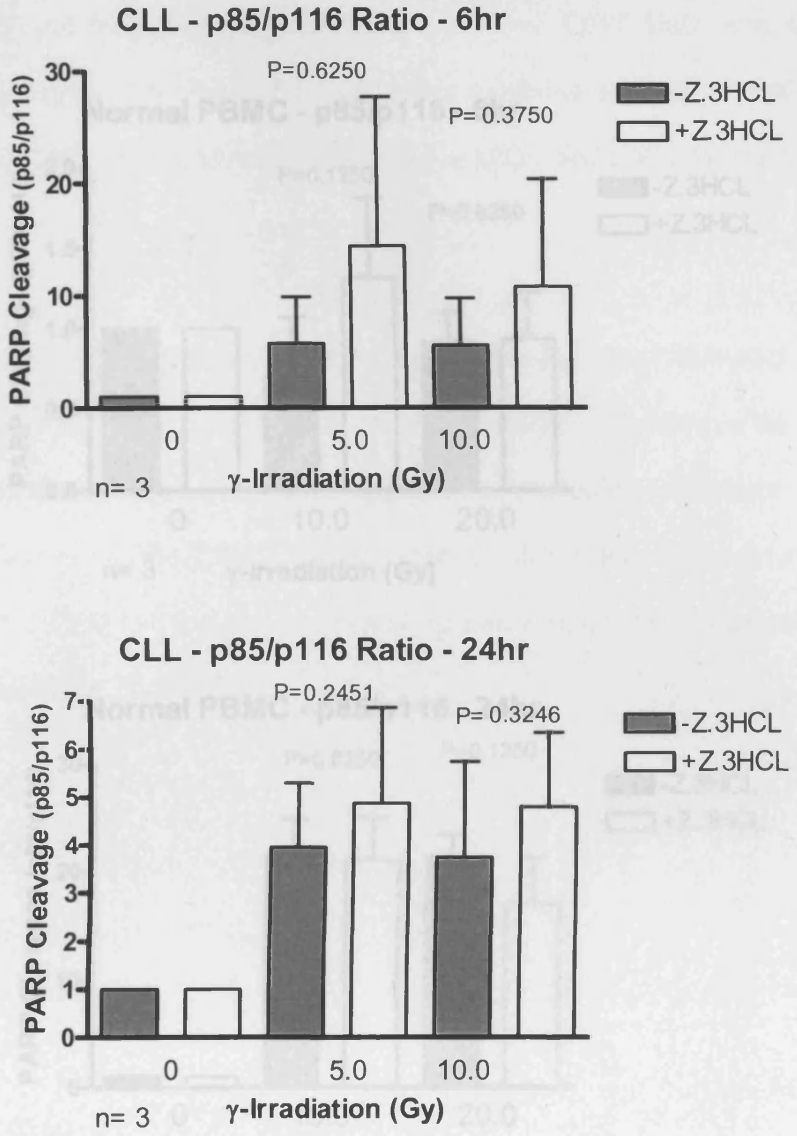
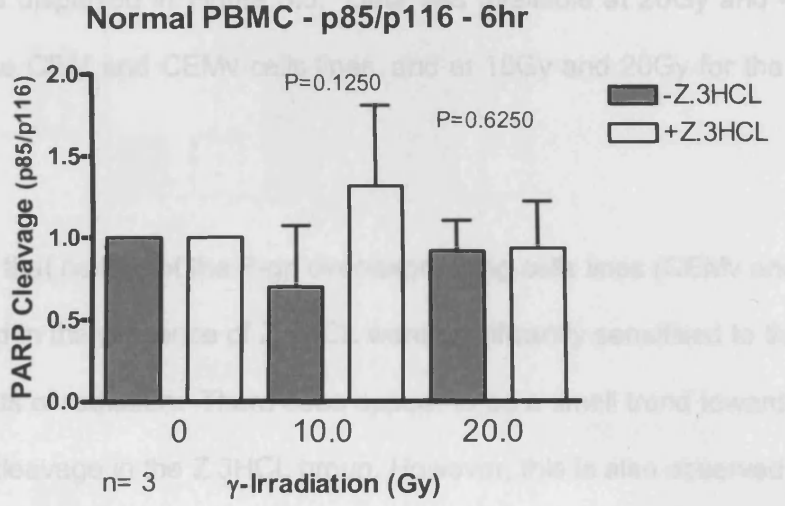


Figure 5.3 Western blot apoptosis data for CLL cells treated with radiation. P-gp$^+$ CLL cells exposed to 5Gy and 10Gy of γ-radiation and incubated with and without 100nM Z.3HCL for 6 and 24hrs. The results were normalised ratiometrically against the non-irradiated control. Paired t-test performed to determine significance of any sensitisation to apoptosis that may be caused by the P-gp inhibition.



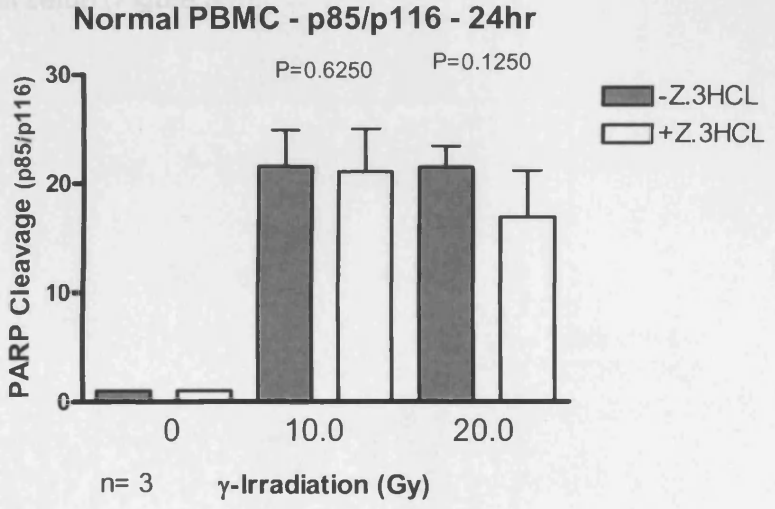


Figure 5.4 Western blot apoptosis data for normal PBMCs. PBMCs isolated from normal donors were exposed to 10Gy and 20Gy of γ-radiation and incubated with and without 100nM Z.3HCL for 6 and 24hrs. Paired t-test performed to determine significance of any sensitisation to apoptosis that may be caused by the P-gp inhibition.

PARP cleavage Western blot data from the P-gp over-expressing cell lines CEMv and VLB and their P-gp negative parent cell line, CEM, were analysed and the results displayed in Figure 5.5. Data was available at 20Gy and 40Gy radiation for the CEM and CEMv cells lines, and at 10Gy and 20Gy for the VLB cells.

It can be seen that neither of the P-gp over-expressing cells lines (CEMv and VLB) incubated in the presence of Z.3HCL were significantly sensitised to the apoptotic effects of radiation. There does appear to be a small trend towards higher PARP cleavage in the Z.3HCL group. However, this is also observed in the P-gp negative CEM cell line and must be concluded that it is an artefact of the experimental setup (Figure 5.5).

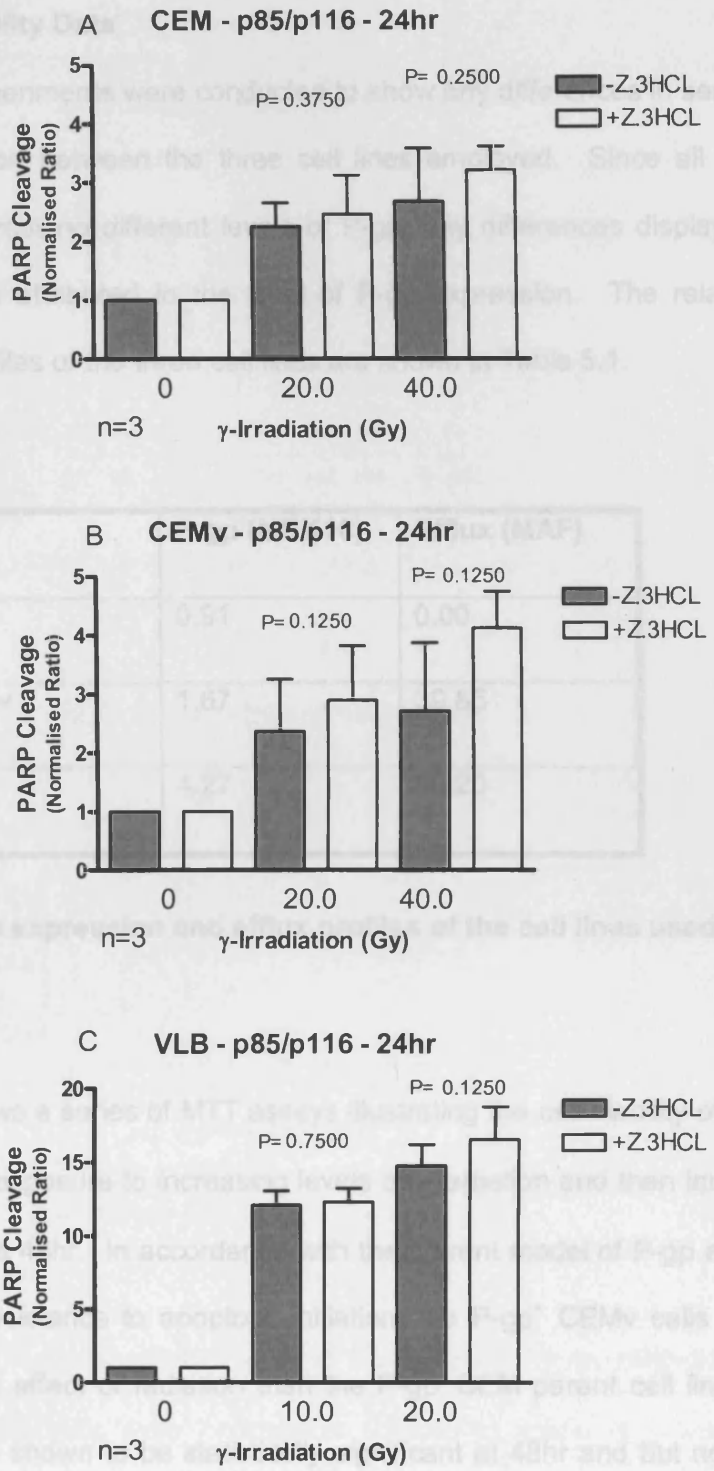


Figure 5.5 Western blot apoptosis data for CEM, CEMv and VLB cells treated with radiation. Cells were exposed to 10, 20 or 40Gy of γ-radiation and incubated with and without Z.3HCL for 24hrs. A= CEM, B= CEMv, C= VLB.

5.2.2 Cell Viability Data

A series of experiments were conducted to show any differences in sensitivity to ionising radiation between the three cell lines employed. Since all three are CEM cells expressing different levels of P-gp, any differences displayed could theoretically be attributed to the level of P-gp expression. The relative P-gp expression profiles of the three cell lines are shown in Table 5.1.

	P-gp (MRK16)	Efflux (MAF)
CEM	0.91	0.00
CEMv	1.67	39.85
VLB	4.27	96.20

Table 5.1 P-gp expression and efflux profiles of the cell lines used.

Figure 5.6 shows a series of MTT assays illustrating the cell viability of the three cell lines after exposure to increasing levels of γ-radiation and then incubated at 37°C for 24 and 48hr. In accordance with the current model of P-gp expression bestowing a resistance to apoptotic initiation, the P-gp$^+$ CEMv cells are more resistant to the effect of radiation than the P-gp$^-$ CEM parent cell line. These differences are shown to be statistically significant at 48hr and but not at 24hr. Unexpectedly, the very highly P-gp expressing VLB cells show a greater sensitivity to the apoptotic effects of radiation compared to both the CEM and CEMv cells. (Figure 5.6).

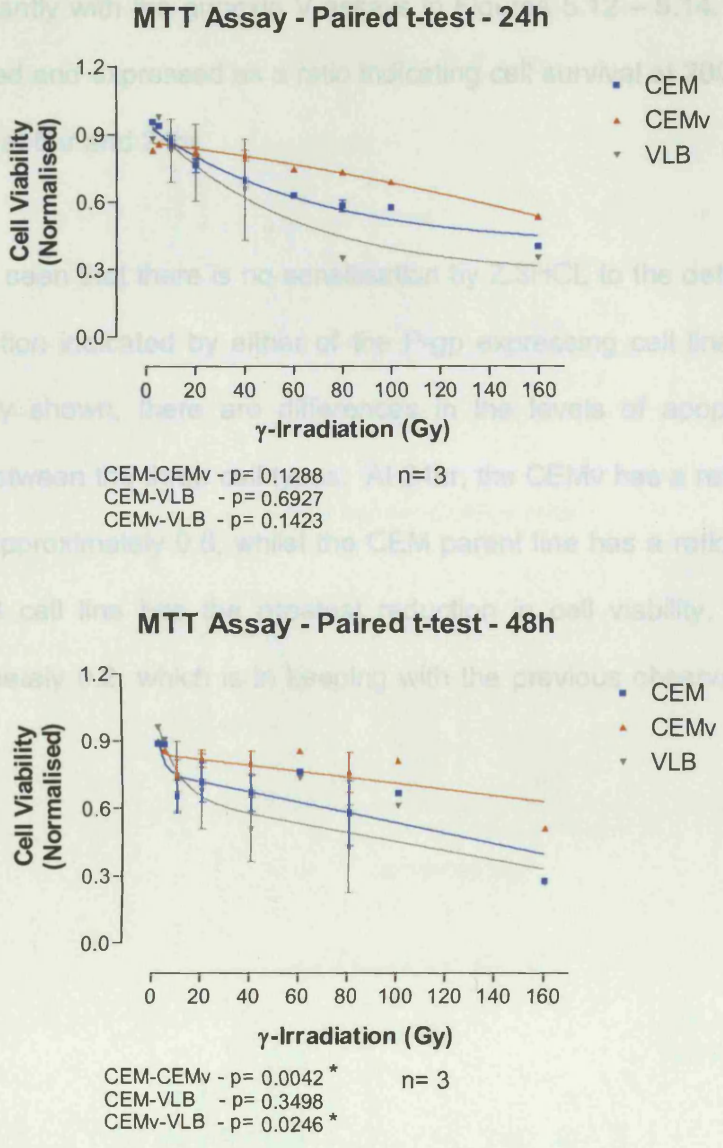
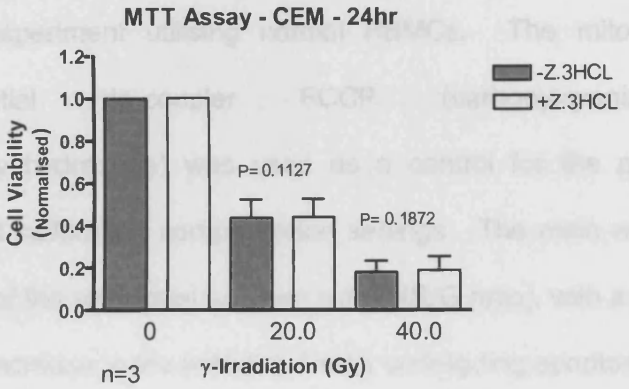
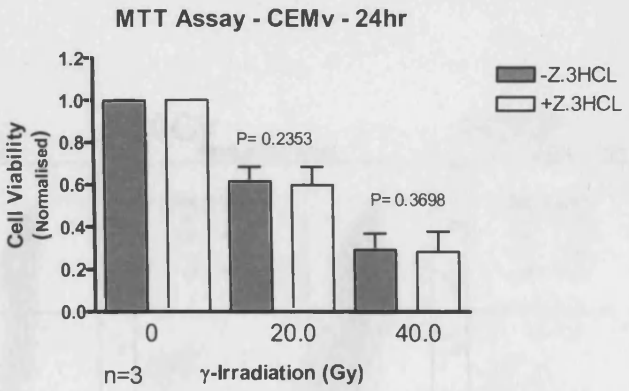


Figure 5.6 MTT assay comparing cell line sensitivity to radiation. CEM, CEMv, and VLB cells were exposed to increasing levels of γ-radiation and incubated for 24hrs and 48hrs. Paired non-parametric tests were performed to determine the significance of any differences in sensitivity between the cell lines. Non-linear regressions were also employed to illustrate the trend in decreasing cell viability.

Figure 5.7 illustrates the results from MTT cell viability assays, conducted concomitantly with the annexin V assays in Figures 5.12 – 5.14. The data was normalised and expressed as a ratio indicating cell survival at 20Gy and 40Gy of radiation at 6hr and 24hr.

It can be seen that there is no sensitisation by Z.3HCL to the detrimental effects of irradiation indicated by either of the P-gp expressing cell lines, although as previously shown, there are differences in the levels of apoptotic sensitivity shown between the three cell types. At 24hr, the CEMv has a relative apoptosis ratio of approximately 0.6, whilst the CEM parent line has a ratio of around 0.4. The VLB cell line has the greatest reduction in cell viability, with a ratio of approximately 0.2, which is in keeping with the previous observations in Figure 5.6.





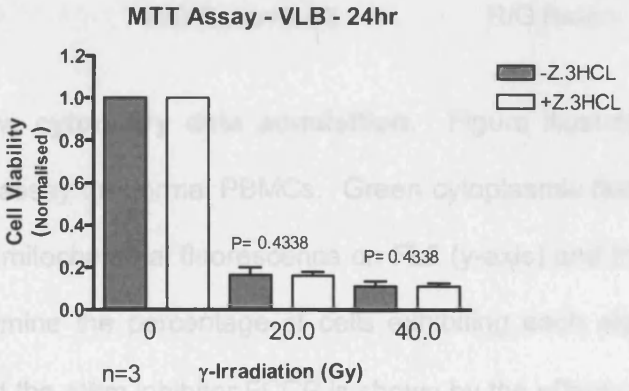


Figure 5.7 MTT Assay with irradiated CEM, CEMv and VLB cells. Cells exposed to between 0Gy, 20Gy and 40Gy of radiation and incubated with and without 100nM Z.3HCL for 6hr and 24hr. Paired statistical analysis was performed to establish any significance between groups

5.2.3 JC-1 Assay Data

Figure 5.8 illustrates the flow cytometry acquisition strategy employed for the JC-1 assay from an experiment utilising normal PBMCs. The mitochondrial membrane potential de-coupler FCCP (carbonylcyanide p-trifluoromethoxyphenyl-hydrazone) was used as a control for the placing of acquisition gates and calibrating compensation settings. The main results are reported as the ratio of the red signal to green signal (R/G ratio), with a decrease in ratio signifying an increase in the amount of cells undergoing apoptosis.

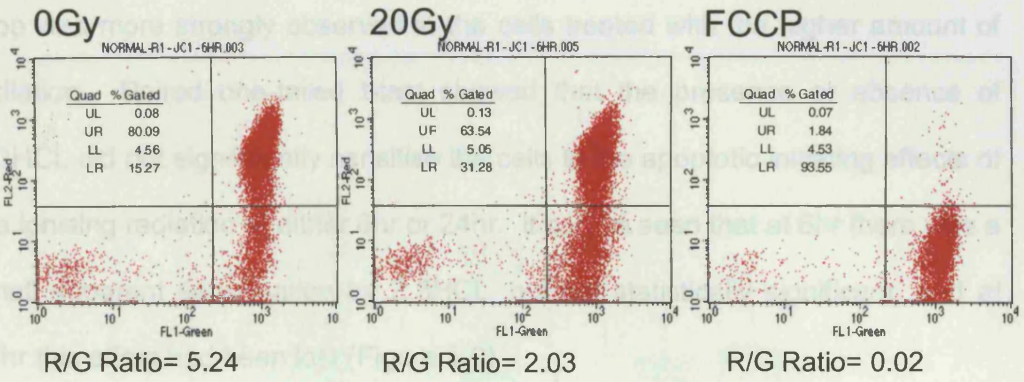
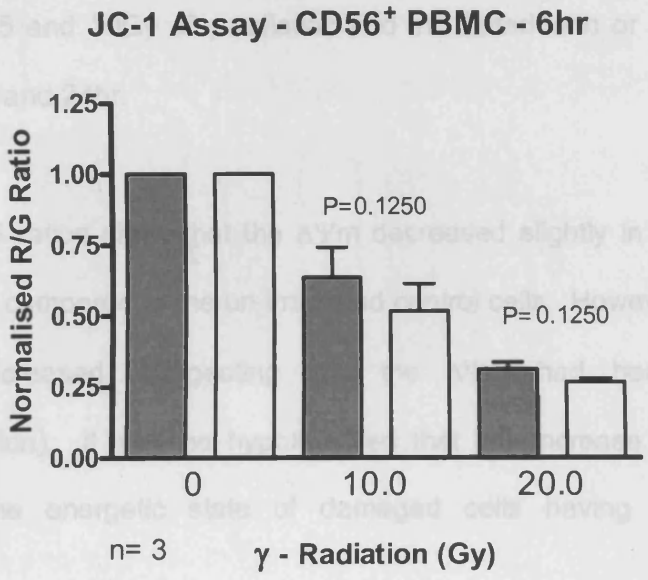


Figure 5.8 JC-1 flow cytometry data acquisition. Figure illustrating basic principle of the JC-1 assay on normal PBMCs. Green cytoplasmic fluorescence on FL1 (x-axis); Red mitochondrial fluorescence on FL2 (y-axis) and the dot plot is quartered to determine the percentage of cells exhibiting each signal. The effect of radiation and the $\Delta\Psi m$ inhibitor FCCP is shown by the effect on the R/G signal ratio.

Figure 5.9 shows the results of a JC-1 assay on γ-irradiated normal $CD56^+$ PBMCs, in an attempt to utilize the inherently high P-gp status of $CD56^+$ NK cells as a means of investigating the effect of P-gp expression on apoptosis. Prior MDR analysis confirmed that the cells isolated from the normal donors were $P\text{-}gp^+$ (median MRK16= 1.19, range 1.16 – 1.24; median MAF= 15.88, range 13.43 – 16.44). The cells were exposed to 10Gy and 20Gy of γ-radiation and then incubated with and without 100nM of Z.3HCL for 6hr and 24hr.

The R/G ratios revealed that the $\Psi\Delta m$ indicating early apoptotic events, dropped in response to the γ-radiation at both the 6hr and 24hr time points and that the drop was more strongly observed in the cells treated with the higher amount of radiation. Paired one-tailed t-test showed that the presence or absence of Z.3HCL did not significantly sensitise the cells to the apoptotic initiating effects of the ionising radiation at either 6hr or 24hr. It can be seen that at 6hr there was a small apparent sensitisation by Z.3HCL, but not statistically significant, and at 24hr this effect had been lost (Figure 5.9).



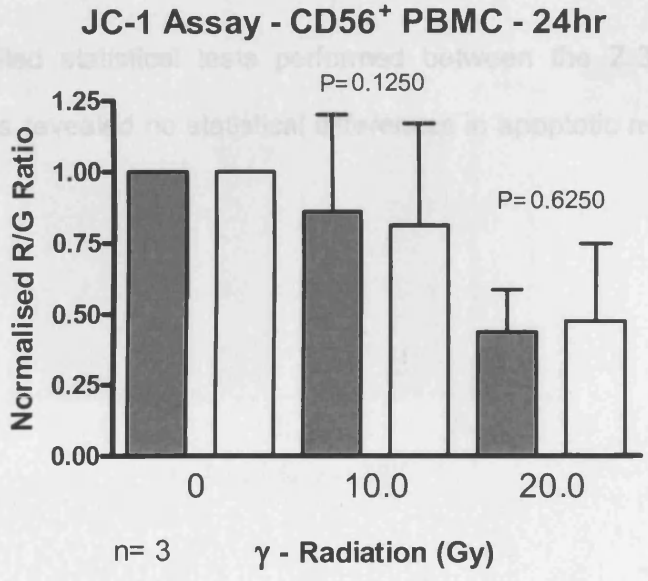
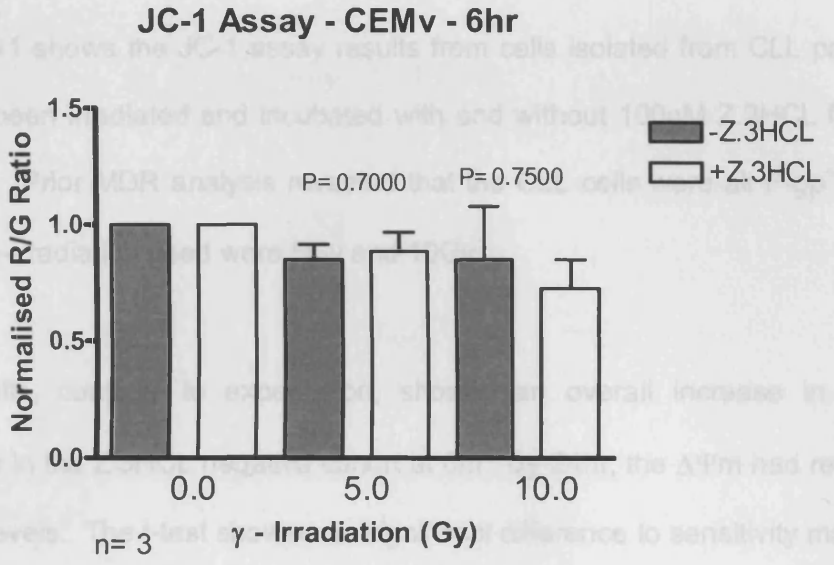


Figure 5.9 JC-1 Assay with irradiated NK cells. $CD56^+$ gated PBMCs isolated from normal donors were exposed to 10Gy and 20Gy of radiation and incubated with and without 100nM Z.3HCL for 6hr and 24hr. Paired t-tests were also performed to establish any significant apoptotic sensitisation.

Figure 5.10 illustrates the JC-1 assay results from the P-gp$^+$ CEMv cell line, exposed to 0, 5 and 10Gy of γ-radiation and incubated with or without 100nM Z.3HCL for 6hr and 24hr.

At 6hr the R/G ratios show that the $\Delta\Psi m$ decreased slightly in response to γ-radiation when compared to the un-irradiated control cells. However, by 24hr the ratios had increased, suggesting that the $\Delta\Psi m$ had become stronger (hyperpolarisation). It may be hypothesised that this increase may reflect an increase in the energetic state of damaged cells having initiated repair mechanisms.

Paired, one-tailed statistical tests performed between the Z.3HCL and non-Z.3HCL cohorts revealed no statistical differences in apoptotic response (Figure 5.10).



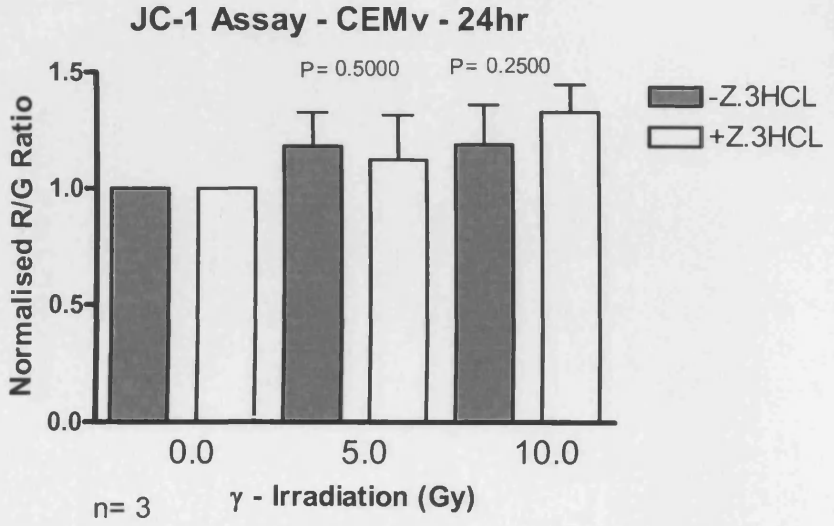
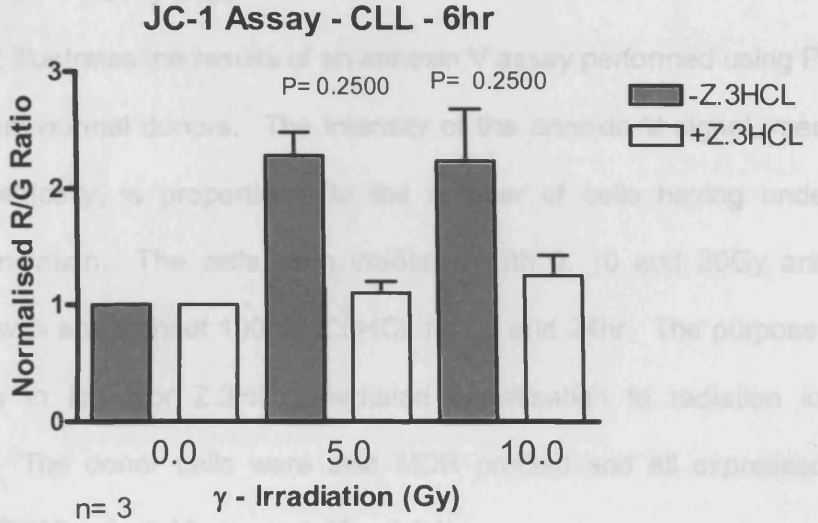


Figure 5.10 JC-1 Assay with irradiated CEMv cells. CEMv cells were exposed to 5Gy and 10Gy of radiation and incubated with and without 100nM Z.3HCL for 6hr and 24hr. Paired t-tests were also performed to establish any significant apoptotic sensitisation.

Figure 5.11 shows the JC-1 assay results from cells isolated from CLL patients that had been irradiated and incubated with and without 100nM Z.3HCL for 6hr and 24hr. Prior MDR analysis revealed that the CLL cells were all P-gp^{+}. The levels of γ-irradiation used were 5Gy and 10Gy.

The results, contrary to expectation, showed an overall increase in $\Delta\Psi m$, especially in the Z.3HCL negative cohort at 6hr. By 24hr, the $\Delta\Psi m$ had returned to basal levels. The t-test showed no significant difference to sensitivity made by the addition of Z.3HCL (Figure 5.11).



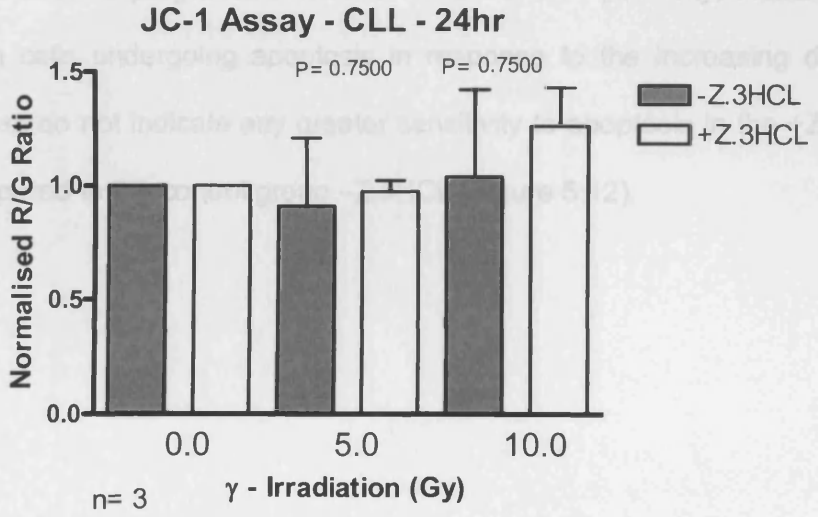
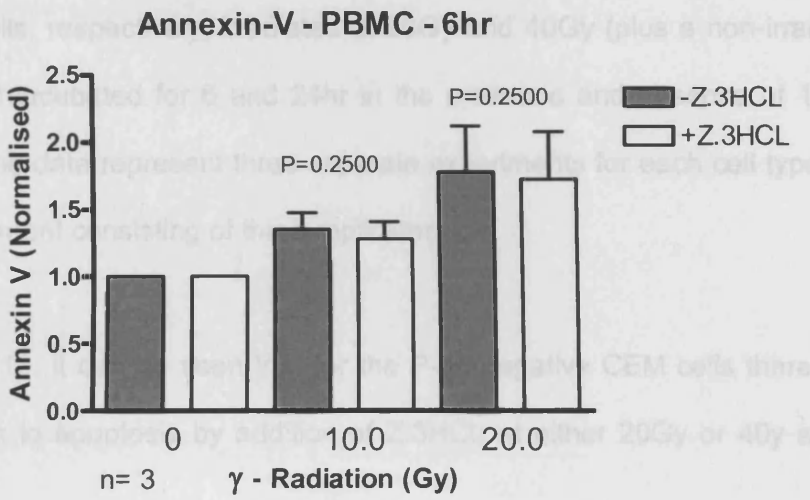


Figure 5.11 JC-1 Assay with irradiated CLL cells. CLL cells isolated from patients were exposed to 5Gy and 10Gy of radiation and incubated with and without 100nM Z.3HCL for 6hr and 24hr. Paired t-tests were performed to establish any significant apoptotic sensitisation.

5.2.4 Annexin V Assay Data

Figure 5.12 illustrates the results of an annexin V assay performed using PBMCs isolated from normal donors. The intensity of the annexin V signal, measured flow-cytometrically, is proportional to the number of cells having undergone apoptotic initiation. The cells were irradiated with 0, 10 and 20Gy and then incubated with and without 100nM Z.3HCL for 6h and 24hr. The purpose of the assay was to look for Z.3HCL mediated sensitisation to radiation induced apoptosis. The donor cells were also MDR profiled and all expressed P-gp (median MRK16 ratio 1.19, range 1.16 – 1.24).

The results show a progressive increase in annexin V positivity, indicating an increase in cells undergoing apoptosis in response to the increasing dose of radiation, but do not indicate any greater sensitivity to apoptosis in the +Z.3HCL group compared to the control group –Z.3HCL (Figure 5.12).



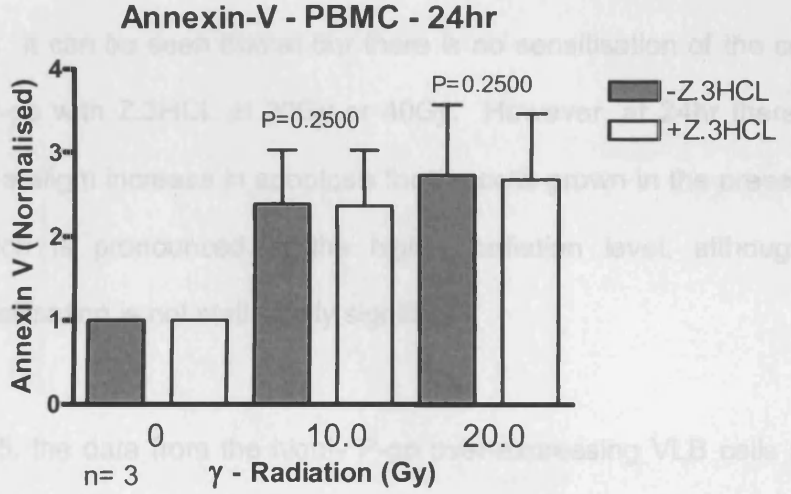


Figure 5.12 Annexin V Assay with irradiated PBMCs. PBMCs isolated from normal donors exposed to 10Gy and 20Gy of radiation and incubated with and without 100nM Z.3HCL for 6hr and 24hr. Paired t-tests were performed to establish any significant apoptotic sensitisation.

Figures 5.13, 5.14 and 5.15 show the annexin V assay results in CEM, CEMv and VLB cells, respectively, irradiated at 20Gy and 40Gy (plus a non-irradiated control) and incubated for 6 and 24hr in the presence and absence of 100nM Z.3HCL. The data represent three separate experiments for each cell type, and each experiment consisting of three replicates.

In Figure 5.13, it can be seen that for the P-gp negative CEM cells there is no sensitisation to apoptosis by addition of Z.3HCL at either 20Gy or 40y at both time points.

Figure 5.14 shows the results for the CEMv cells, which express an intermediate level of P-gp. It can be seen that at 6hr there is no sensitisation of the cells by blockading P-gp with Z.3HCL at 20Gy or 40Gy. However, at 24hr there does appear to be a slight increase in apoptosis for the cells grown in the presence of Z.3HCL, which is pronounced at the higher radiation level, although this apparent sensitisation is not statistically significant.

In Figure 5.15, the data from the highly P-gp over-expressing VLB cells shows an apparent sensitisation in the Z.3HCL group at 6hr for both radiation points, but this difference is not statistically significant. At 24hr, the levels of sensitisation appear more pronounced at both radiation levels than at 6hr, and this difference reaches statistical significance at the 40Gy point (P=0.0367), indicating that the P-gp modulatory effect of Z.3HCL had sensitised the VLB cells to apoptosis.

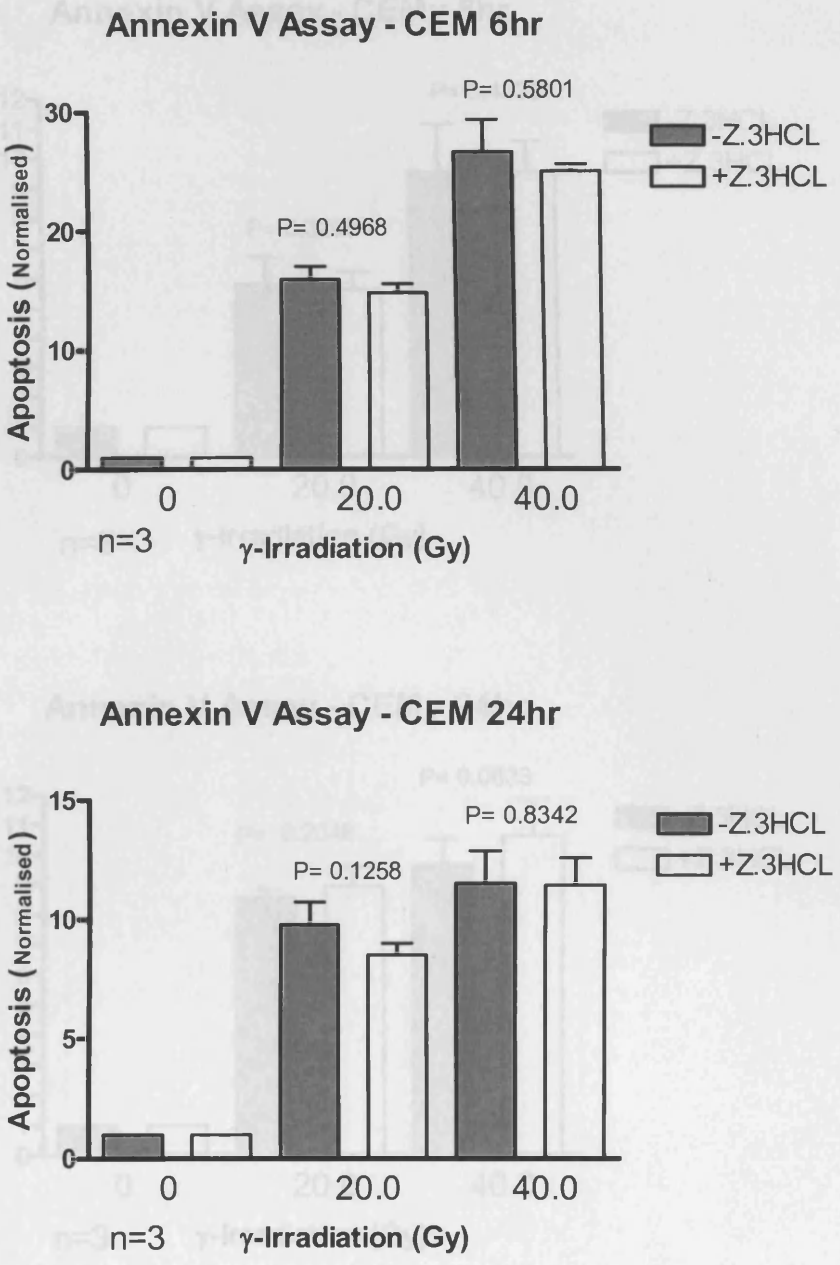
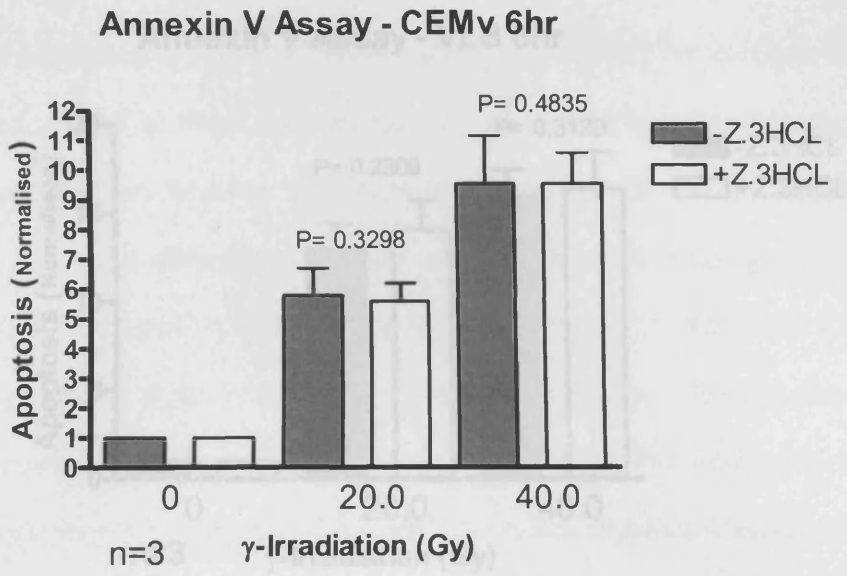


Figure 5.13 Annexin V Assay with irradiated CEM cells. CEM cells exposed to 0Gy, 20Gy and 40Gy of radiation and incubated with and without 100nM Z.3HCL for 6hr and 24hr. The results were normalised ratiometrically against the non-irradiated control. Paired statistical analysis was performed to establish any significant apoptotic sensitisation.



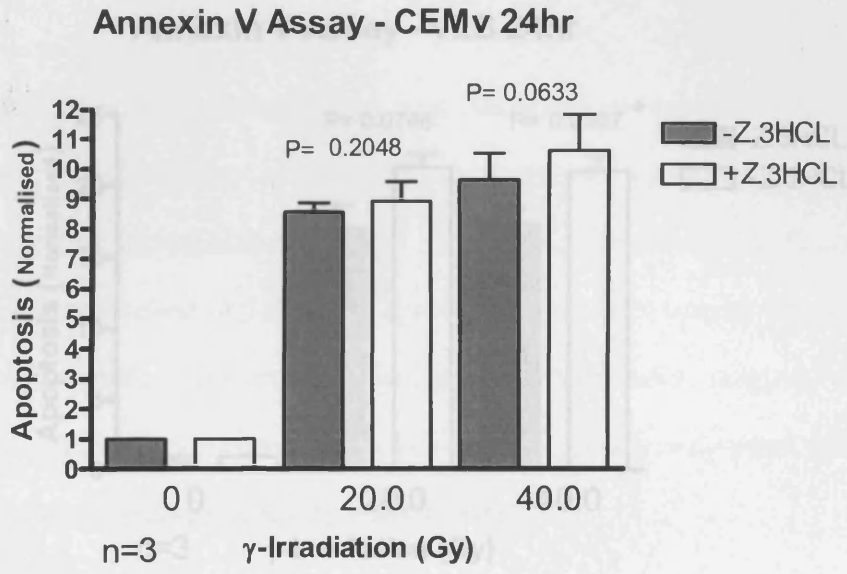
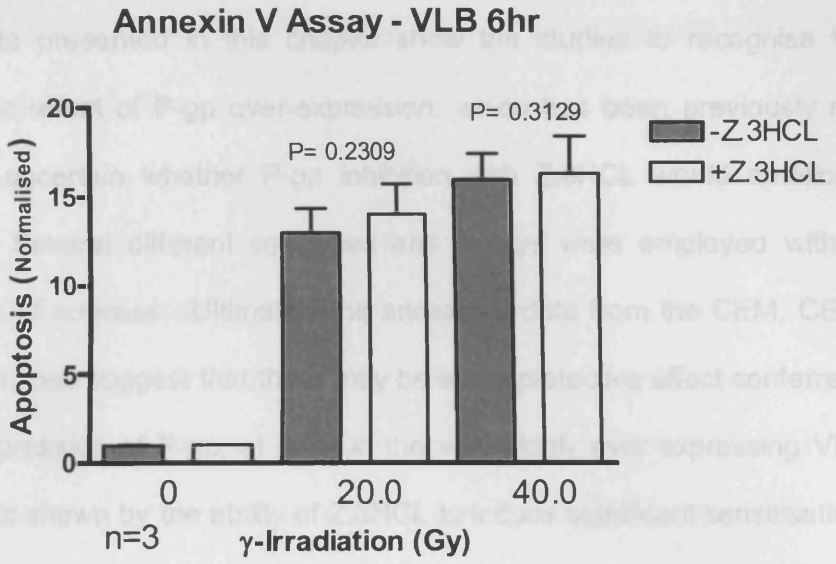


Figure 5.14 Annexin V Assay with irradiated CEMv cells. CEMv cells exposed to 0Gy, 20Gy and 40Gy of radiation and incubated with and without 100nM Z.3HCL for 6hr and 24hr. Paired statistical analysis was performed to establish any significant apoptotic sensitisation.



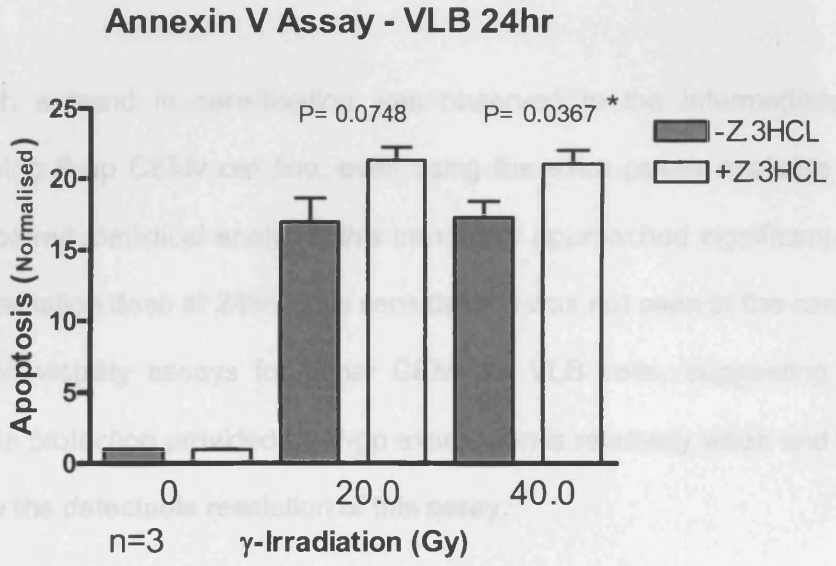


Figure 5.15 Annexin V Assay with irradiated VLB cells. VLB cells exposed to 0Gy, 20Gy and 40Gy of radiation and incubated with and without 100nM Z.3HCL for 6hr and 24hr. Paired statistical analysis was performed to establish any significant apoptotic sensitisation.

5.3 Discussion

The data presented in this chapter show the studies to recognise the anti-apoptotic effect of P-gp over-expression, which has been previously reported, and to ascertain whether P-gp inhibition with Z.3HCL would ameliorate this effect. Several different cell types and assays were employed with varying degrees of success. Ultimately the annexin V data from the CEM, CEMv, and VLB cell lines suggest that there may be some protective effect conferred by the over-expression of P-gp, at least in the very highly over expressing VLB cells. This was shown by the ability of Z.3HCL to induce significant sensitisation in the VLB cells, indicating that P-gp was in some way responsible for increasing the VLB cells apoptotic threshold, and that inhibiting P-gp ameliorated this effect.

Although a trend in sensitisation was observed in the intermediately over-expressing P-gp CEMv cell line, even using the extra power available with the use of paired statistical analysis, this trend only approached significance for the higher radiation dose at 24hr. This sensitisation was not seen in the concomitant MTT cell viability assays for either CEMv or VLB cells, suggesting that the apoptotic protection provided by P-gp expression is relatively weak and its effect is below the detectable resolution of this assay.

For most of the experiments conducted in this chapter, ionising radiation was used as an inducer of apoptosis, which had its advantages and disadvantages when compared to the use of cytotoxic drugs. One significant advantage was the guarantee of null drug efflux effects causing a bias when using P-gp expressing cells. A disadvantage was that although γ-radiation causes apoptosis, it also causes senescence, meaning that even though P-gp may

protect against apoptosis the employed indicators of this, especially the cell viability assay, may miss it because of other forms of cell cycle arrest that radiation induces.[332]

The MTT cell viability assays conducted on the three CEM cell lines (CEM, CEMv, VLB) showed, contrary to initial expectation, that the P-gp over-expressing VLB cell line was more sensitive to radiation than the P-gp negative parent CEM line. Earlier studies by Matarrese *et al* also shows that the VLB sub-line was more sensitive to some apoptotic stimuli, specifically TNF-α, than the parent CEM cell line[333].

It may be hypothesised that the very high levels of P-gp expression by the VLB cells could in some way undermine the viability of these cells. It could be either through plasma membrane perturbations caused by the density of P-gp protein, or the metabolic cost of producing such a high level of one protein leads to a deficiency in other mechanism of cell fitness. This explanation would be concordant with the fact that radiation can also kill cells via non-apoptotic catastrophic cell death mechanisms, and that any disruption to the cell membrane could feasibly strengthen this effect[332].

The Matarrese study, however, noted that there was a correlation between high levels of P-gp expression and increased mitochondrial transport chain activity, presumably due to the basal ATPase activity of P-gp and the high energy requirements that would entail in a cell line such as VLB. In addition, it was also found that the increase in mitochondrial activity was also accompanied by perturbations in the lipid and protein composition of the mitochondrial

membrane[334]. It has already been show that a reduced mitochondrial electron transport chain activity in leukaemic cells was associated with an apoptotic protective effect. It was therefore hypothesised that the converse may also be true, and was indeed demonstrated by the experimental use of compounds known to suppress electron transport chain activity[333]. This suggests that the reason for the increased sensitivity to radiation observed in the VLB may be due to the increased energy demands of the high P-gp phenotype, and the concomitant changes to the mitochondria membrane. This in turn may be associated with an increased sensitivity to the initiation of apoptosis via the release of cytochrome c from the mitochondrial membrane, in response to certain apoptotic stimuli, such as ionising radiation[335-338].

In contrast, the CEMv cell line, which over expresses P-gp to an intermediate level compared to CEM and VLB cells was found to be significantly more resistant to the effects of γ-radiation than the parent cell line CEM. Incubating the CEMv cells with Z.3HCL caused the cells to become more sensitive to radiation, suggesting that the protective effect was conferred by the expression of P-gp.

Western blots were used initially to probe for PARP cleavage, a well established method of observing apoptosis[339,340]. This technique is very time and labour intensive and we were keen to employ flow-cytometric technology, not least because of the rapidity of data acquisition. Ultimately, the western blot data did not show any evidence of apoptotic sensitisation by Z.3HCL for the CEM, CEMv or VLB cells.

The JC-1 assay was employed in an attempt to look for mitochondrial changes that might signify apoptotic initiation[341]. JC-1 has previously been investigated as a fluorescent probe for use in MDR profiling assays in AML cells because it is an avid substrate for P-gp efflux[315,316]. In our experience, although JC-1 proved itself to be extremely sensitive as an indicator of P-gp expression, the technical difficulties introduced by having to calibrate the flow cytometer compensation for a biphasic fluorophore meant that it was unsuitable for routine use, especially since the relatively simple and reliable calcein-AM assay was already established. These technical difficulties, especially that intracellular concentrations of JC-1 are so drastically affected by P-gp efflux, also hindered the studies into the role of P-gp expression in apoptosis. Until a reliable means of calibrating the compensation setting are established its use in this area may be marginalised. Repeated experiments showed that the mitochondrial membrane inhibitor FCCP was the most consistent means of establishing a baseline reading for the assay.

In the JC-1 assay, normal $CD56^+$ cells were utilised because of their high P-gp status. The functional requirement of P-gp expression on haematological cells is uncertain and may be directly linked to a dampening effect of the apoptotic response as a way of protecting against bystander killing in areas of cell-mediated immune response. P-gp may serve to protect immune cells against stress-induced apoptosis or bystander lysis mediated by either the Fas or TNF death pathways in the hostile site of inflammation. In addition, it has been suggested that P-gp could play a role in the growth and/or survival of primitive haemopoietic stem cells[180]. However, this assay did not show any significant

difference in sensitivity for NK cells incubated in the presence or absence of Z.3HCL.

Data from the annexin V assays (Figures 5.12 – 5.14), in conjunction with 1-tailed, paired analysis, at best suggested that there was a small Z.3HCL induced sensitisation to apoptosis in irradiated CEMv cells (approaching significance, P=0.0633) and VLB cells (significant, P=0.0367) which was not apparent on the CEM cells. This trend was not observed in the normal PBMCs, which were P-gp$^+$, nor in concomitant MTT assays.

The annexin V assay was employed after the technical difficulties experienced with the JC-1 assay since neither the FITC-annexin V conjugate nor the PI were affected by P-gp expression. In addition, because the two fluorescent signals were generated from two different agents, the compensation procedure was much simplified. The annexin V assay proved itself to be extremely sensitive and in this study was deemed to provide the most consistent data.

In summary, the data from the CEM, CEMv and VLB cell lines suggest that P-gp expression has a significant (VLB) or nearing-significant (CEMv) protective effect upon the initiation of apoptosis, when compared to the basal level for that cell line, and that this effect can be ameliorated by use of the P-gp modulating agent Z.3HCL. This effect was not observed in the CLL cells and normal PBMCs, both of which over-expressed P-gp, albeit at a considerably lower level than CEMv and VLB.

6. Final Discussion

6.1 Discussion

It has been shown by many studies that the over-expression of P-gp in cancer is an indicator of poor prognosis and chemotherapeutic treatment failure, and this is particularly so for the haematological tumours such as AML, ALL, MM and CML[329,342,343]. Different studies have apportioned varying levels of importance to what P-gp expression means to prognosis and the impact on the achievement of complete remission, relapse rates, and overall survival[309,344].

The use of chemical adjuvants to standard chemotherapy in an attempt to overcome the resistance effects of P-gp have so far shown mixed results, with little or no obvious benefit overall, except for possibly those patients who do indeed over-express P-gp. These early trials have been typically conducted on difficult patient groups such as the elderly, relapsed and refractory, all categories associated with P-gp over-expression. Additionally, only first and second generation inhibitors whose toxicity and specificity profiles fall somewhat short of those exhibited by the current range of third generation compounds[345,346] have been used.

It can be seen that the relationship between P-gp expression and MDR function is not always a direct one. The flow cytometry data presented in chapters 3 and 4 indicate that even the measured expression of P-gp does not necessarily indicate measurable efflux function. Neither does efflux function guarantee measurable P-gp expression, although there is an overall correlation. The classical model of simple P-gp upregulation in response to chemotoxic insult falls somewhat short of the mark and must be reconsidered in light of the highly

convoluted means of P-gp induction, activation and suppression at the nuclear, cytoplasmic and lipid level[159,347,348]. This model is further complicated in light of the fact that both normal and leukaemic stem cells are characterised by their high level of inherent MDR status[349,350].

The fluorescent dye efflux data in chapters 3 and 4 show Z.3HCL to be a potent P-gp inhibitor in patients with AML, and other haematological malignancies. R123 efflux from $CD56^+$ cells was rapidly inhibited in all of the patients studied, often within an hour from the start of infusion. This trend was also observed for the $CD33^+$ cells in the AML clinical trial. However, $CD33^+$ cells tend to express significantly less P-gp than $CD56^+$ cells and the inhibitory effect was therefore less pronounced.

As the previous clinical trials of P-gp inhibitors suggest, specific targeting of only the P-gp over-expressing patients may be the most efficacious strategy to adopt[264,267]. *In vitro* assessment of the patient's MDR status prior to treatment would be therefore necessary, and the assays incorporating calcein-AM and Z.3HCL, as shown by the data presented herein, have proved to be rapid, non-costly and reliable.

It is possible, by using both efflux function and expression to score semi-quantitatively a patient's cells for P-gp expression based on whether they are double positive or negative for both measures or positive for one or the other. This approach can then also be augmented by considering the drug sensitivity profile obtainable from MTT assays. This approach, as detailed in chapter 4, was seen to be more successfully concordant for the AML cells than CLL cells

and MM cells. This may be because P-gp, although often expressed on CLL cells, has not been shown to be a major mechanism for drug efflux resistance[351], where in AML it has been consistently shown to be a significant factor[183,352]. The technical difficulties experienced in obtaining sufficient numbers of MM plasma cells ultimately curtailed the scope of the experiments that could be conducted, thus reducing the analytical power available.

The safety profile data, from mainly the AML trial but also contributed to by the two solid tumour trials, do suggest that Z.3HCL can be used along side conventional chemotherapy with acceptable risk. Indeed, there is evidently some scope for adjusting the administration regimen to minimise onset of these deleterious toxicities. Overall, Z.3HCL has two main advantages over previous modulators in that it has very little effect on the clearance of the coadministered drugs and their metabolites, such as daunorubicin and daunorubicinol. Also it does not affect the cytochome P450 isoenzymes such as CYP3A at administered doses, which would potentially lead to increasing the toxicity profile of the drug regimen[318,353]. However, it may also be argued that because it has been shown that ABC transporters can be co-expressed, and may even act in concert, that a new generation of inhibitor, with an inherently better safety profile, may have an advantage over a single specificity compound such as Z.3HCL[354].

The role of P-gp in apparently protecting cells from apoptosis is still very much an area of ongoing research. Although several proof of principle studies have shown that there is an effect, including some of the data presented in Chapter 5, its exact mechanism of action is yet to be elucidated along with whether this effect is of any clinical relevance, especially since the majority of the previous

research has been conducted on cell lines[109,180,182,355,356]. Pallis *et al* have shown that P-gp expression on AML blasts has a protective effect against spontaneous apoptosis in culture and that studies with cell lines have linked this to P-gp involvement with sphingomyelin and ceramide trafficking[184,357].

The annexin V results from Chapter 5 suggest that Z.3HCL can be used for both the detection and modulation of the apoptotic effect. However, since the effect appears to be small, especially when compared to the drug efflux function, technical difficulties associated with the assays involved may conceal the effect. The moderate P-gp expression of the CEMv cell line did seem to offer a degree of apoptotic protection when compared to the CEM cells, and Z.3HCL did appear to be able to lessen this effect, although not to a statistically significant degree. Paradoxically, the very high level of P-gp expression of the VLB cells was associated with an increased sensitivity to ionizing radiation compared to the CEM, and that Z.3HCL was still associated with a further increase in apoptosis. Studies by Mataresse *et al* suggest that this effect may be linked to the high-energy cost of P-gp and a subsequent vulnerability to cytochrome c release associated with the high level of electron transport chain activity in VLB cells[333].

Any future work pertaining to P-gp modulation in cancer, and in AML in particular, must address the concept of the leukaemic hierarchy and the importance of the leukaemic stem cell (LSC). In the normal haematopoietic system there are thought to be three distinct populations of progenitor cell: stem cells that have the capacity for long-term renewal; stem cells with the ability for short-term renewal; and multipotent progenitors, which do not have the capacity to renew, but can differentiate into the various cell lineages seen in the bone

marrow[349]. Ultimately, a small number of haematopoietic stem cells (HSCs) retain a capability for self-renewal, which is only conferred to a tightly regulated subset of daughter progeny, whilst other products of HSC cell division differentiate and progress to populate the subsequent levels of the haematopoietic hierarchy.

It has become clear that this hierarchical architecture is paralleled in AML, and that the rapidly proliferating bulk cells that constitute the pathological manifestation of the disease arise as a consequence of the existence of a small number of relatively quiescent LSCs[358]. AML LSCs were found to exist within the $CD34^{+}CD38^{-}$ population of malignant cells, demonstrated by leukaemia initiating engraftment experiments in NOD/SCID mice. These showed that only cells from within this compartment retained the self-renewing potential necessary for leukaemic engraftment[294,359]. These leukaemia initiating stem cells represents between 0.1-1.0% of the AML cell population[360].

A further consideration, from the point of view of drug resistance, is that this population of $CD34^{+}CD38^{-}$ cells, within which the HSCs and LSCs reside, is associated with an abundant expression of MDR associated ABC transporters, particularly P-gp and BCRP. These cells have previously been identified as the so-called side population because of their ability to extrude the fluorescent dye Hoechst 33342, a property exploited cytometrically to enable cell sorting enrichment of stem cells[361,362].

The issue for the current strategy of chemotherapy and adjuvant based MDR modulation is that because the LSCs are both quiescent and are inherently

multidrug resistant, agents that exert their chemotherapeutic effect by targeting rapidly proliferating cells are ultimately only dealing with the symptoms and not the cause of the malignancy. The LSC can survive chemotherapy as minimal residual disease and carry on to seed a new generation of malignant disease, which ultimately manifests as clinical relapse[360,363]. Future therapeutic strategies seeking to address this will not only have to target specifically the LSCs, but also to avoid undue damage to normal HSCs, since they share many phenotypical characteristics[293].

One agent that has shown promise in this area is the sesquiterpene lactone parthenolide. Derived from the plant feverfew it is a potent inhibitor of NF-kB, which is constitutively activated in LSCs but not in HSCs. It is not a known P-gp substrate, and it has been shown to preferentially induce apoptosis in LSCs in AML, CLL and CML, whilst leaving normal HSCs intact[364,365]. Other, future compounds directed against the LSC may well also be P-gp substrates, in which case there will still be a role for MDR inhibitors as adjuvants to chemotherapy.

6.2 Further Work

1) Further refinement of the *in vitro* assays, especially the incorporation of a blast marker such as anti-CD45RA, to enable a more accurate assessment of patient tumour P-gp status. Recently, the French Drug Resistance Network refined and improved their standard method for P-gp detection in haematological malignancies. Their revised methodology claims an increased sensitivity and specificity for both antibody detection and functional analysis, and these revisions have been validated by the various laboratories that participated within

the network[366]. In light of this, it could be suggested that any further investigation into this field, in the interest of compatibility of data, should consider the adoption of this methodology. However, the calcein-AM based assay employed throughout the bulk of this study has proved itself to have many advantages in terms of cost, speed, sensitivity and easy of use. It could almost certainly be used, in conjunction with extra cell population defining modifies such as anti-CD45 and propidium iodide.

2) Clinically, the administration regimen of Z.3HCL should be optimised to minimise the toxicity experienced without affecting its efficacy as a P-gp inhibitor. In light of the outcomes of the previous phase III clinical trials, a targeted approach should probably be adopted for the inclusion of a P-gp inhibitor, since data from several of the trials suggest that only patients who show an upfront overexpression of P-gp will gain benefit[264,267]. A rapid and reliable MDR status screening protocol would therefore be necessary for the pre-treatment assessment of patient samples.

3) To further elucidate the mechanism underlying the role of P-gp in protecting against apoptotic stimulus, and to ascertain whether this effect by itself, or in combination with the drug efflux function is of any clinical relevance. The creation of an inducible transfection cell line model would be useful for measuring the changes in apoptotic sensitivity at differing levels of P-gp expression, and measuring the cut off point, as implied by the CEMv and VLB cells, whereby P-gp expression ceases to exert a net protective effect and acts as a sensitizer to certain apoptotic stimuli. To establish the likelihood of any

clinical relevance, primary AML cells would have to be used in short-term culture in sufficient numbers to establish whether there is any statistically significant difference in apoptotic sensitivity between P-gp overexpressing and P-gp negative blast cells.

6.3 Conclusions

Z.3HCL has proved to be both potent and specific as an inhibitor of P-gp, both *in vivo* in the clinical setting, and *in vitro* at the bench level. It has a relatively low toxicity profile and has been shown to be safe to use as an adjuvant with several standard chemotherapy regimens. It is able to inhibit potently both the classical drug efflux function and possibly the proposed apoptotic suppression function of P-gp without itself being a substrate for efflux. These attributes recommend further study into its use as a targeted therapy for P-gp engendered MDR in haematological malignancies.

7. References

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Appendix

1. AML Patient Data

Patient	Sex	Age	FAB	P/R/T	P/B	%Blast (BM)	WBC	MAFz	MAFv	MRK16	Cyto	Rx	Response
1	F	59	n/a	n/a	BM	n/a	n/a	4.40	7.78	0.98	n/a	n/a	n/a
2	M	82	n/a	n/a	PB	n/a	20.2	62.48	70.57	1.88	n/a	n/a	n/a
3	F	35	n/a	P	PB	98	63	6.94	5.25	0.93	t(11q;23)	H-DAT	CR
4	M	63	MDS	n/a	PB	n/a	5.6	11.04	20.15	0.98	n/a	n/a	n/a
5	M	76	n/a	n/a	BM	n/a	4.4	7.76	17.97	1.26	n/a	n/a	n/a
6	M	76	n/a	n/a	PB	n/a	103.8	5.25	19.42	1.11	n/a	n/a	n/a
7	M	76	n/a	n/a	BM	n/a	64.4	1.00	9.40	0.88	n/a	n/a	n/a
8	M	69	n/a	n/a	BM	n/a	128.8	14.45	12.54	1.64	n/a	n/a	n/a
9	M	39	rAML	T	BM	31	10.8	-8.43	12.62	1.00	Failed	FLAG	CR
10	F	44	M5a	P	PB	80	16.9	13.40	15.70	0.86	Complex	DA/AraC/ZHCL	CR
11	M	76	M5b	P	PB	84	242	9.42	0.00	0.91	n/a	n/a	n/a
12	M	31	M5	P	PB	86	6.05	14.18	5.25	0.88	Norm	DA/AraC/ZHCL	CR
13	M	36	M4	P	PB	98	45.4	37.90	39.60	1.36	trisomy22/inv16	DA/AraC/ZHCL	CR
14	M	53	M4	R	PB	36	14.6	3.53	-4.60	0.80	trisomy21	DA/AraC/ZHCL	PD
15	M	62	n/a	n/a	PB	n/a	48.7	1.78	2.66	1.02	n/a	n/a	n/a
16	M	81	M2	P	PB	79	20.5	-3.64	26.99	1.04	n/a	FLAG x2	CR
17	F	72	M0	n/a	PB	n/a	47	45.75	52.17	2.59	n/a	n/a	n/a
18	M	24	M6	P	PB	45	3.5	3.53	12.62	1.08	Norm	DA/AraC/ZHCL	CR
19	M	54	M2	R	BM	86	2.6	-1.82	10.23	0.94	t(6;9)	DA/AraC/ZHCL	CR
20	M	56	M1	P	BM	92	7.1	57.45	27.00	1.14	Norm	DA/AraC/ZHCL	CR
21	F	65	M7	T	PB	15	56	12.62	37.36	1.02	20q	HU	NR
22	M	47	M5a	P	PB	65	5	4.40	18.69	1.07	Norm	DA/AraC/ZHCL	CR
23	F	53	M3	P	BM	43	8.9	-6.50	10.23	1.10	t(15;17)	H-DAT	n/a
24	M	25	M4	R	BM	95	22.8	11.04	25.65	1.04	trisomy 4	DA/AraC/ZHCL	CR
25	M	25	M4	P	BM	90	24	14.18	20.85	0.99	Abnormal	S-DAT	CR
26	M	69	M1	P	PB	80	2.6	0.89	22.26	0.83	Failed	DA/AraC/ZHCL	n/a
27	M	52	M2	P	BM	45	5.2	28.95	35.64	1.35	mono 7	DA/AraC/ZHCL	PD
28	F	71	M2	P	BM	50	1.8	5.25	18.69	1.12	trisomy11/del X	DA/AraC/ZHCL	CR
29	M	63	M4	P	PB	n/a	26.8	8.60	10.23	0.84	Abnormal	DA/AraC	NR
30	F	82	M4	T	PB	50	75.09	1.78	15.71	0.90	11q	HU/Etop	n/a
31	M	65	M2	P	BM	82	5.3	3.53	9.42	0.75	Abnormal	DA/AraC	n/a
32	F	24	M5	P	PB	73	0.7	10.23	11.05	1.22	Failed	ADE	NR
33	F	46	M4	P	PB	50	9.8	47.83	48.42	0.92	Norm	S-DAT	CR
34	M	53	MDS	R	BM	45	1.1	19.41	11.20	1.05	Norm	FLAG	NR
35	F	40	M4	P	BM	56	8.8	-2.86	5.14	0.89	Norm	DA/AraC	CR
36	M	46	M1	R	BM	86	206.4	-11.60	1.27	0.91	n/a	FLAG	CR
37	M	56	M4	P	BM	51	1.23	-5.19	21.81	0.96	n/a	FLAG	NR
38	M	61	M1	P	BM	91	87.8	10.56	21.76	1.06	Norm	DA/AraC/Flag	CR
39	M	44	M1	P	BM	87	10.5	-2	12.43	0.97	Norm	FLAG	CR
40	M	66	M1	P	BM	95	98	9.45	10.46	0.93	Norm	DA/AraC	CR
41	M	11	M5	R	BM	97	5.1	3.44	2.48	1.06	n/a	FLAG	NR
42	M	76	M1	P	PB	98	112.4	18.21	28.13	1.27	Norm	DA/AraC	NR
43	M	63	M2	P	BM	55	n/a	27.00	30.80	1.09	Norm	DA/AraC	CR
43		64	M2	T	BM	10	9.4	1.78	-15.48	0.78			
44	F	36	M2	P	BM	20	7.9	-6.50	16.46	0.91	trisomy 8	DA/AraC/ZHCL	CR
44			M2	T	BM	30	1.7	2.66	14.95	1.24			
45	M	59	M1	P	BM	100	25	51.30	58.58	1.70	Trisomy 21	DAT/FLAG	CR
45			M1	T	PB	n/a	n/a	57.83	44.77	2.33			
46	M	63	M4	R	BM	86	6.6	14.18	35.06	1.60	Norm	DA/AraC/ZHCL	PD
46		64	M4	T	PB	n/a	64	27.01	36.79	2.17			
47	M	68	M1	P	BM	84	1	43.26	43.26	1.50	Norm	DA/AraC/ZHCL	CR
47			M1	R	BM	55	2.4	13.01	20.50	1.22			
48	F	77	M2	P	PB	56	13.5	36.79	27.66	1.05	n/a	HU	n/a
48			M2	T	PB	n/a	17.2	19.79	27.32	1.24			
48			M2	T	PB	n/a	15.9	30.57	33.92	1.23			

Key: n/a = data not available; P = presentation; T = Treated; R = Relapsed; CR = Complete Response; NR = No Response; PD = Progressive Disease; DA = Daunorubicin. The coloured entries denote a positive MDR result.

2. CLL Patient Data

Patient	Sex	Age	Stage	P/T/R	WBC	MAF	MAFv	MRK16	ZAP70	IgVh	Diag	Rx
1	M	70	B	T	48.0	15.71	14.18	1.03	0.59	N-ID	1994	Chl x12; GR
2	M	61	A	T	165.0	17.21	8.6	0.99	0.66	UM	1990	Chl; GR
3	M	73	A	UnRx	37.0	19.05	-4.7	3.17	0.67	UM	1988	UnRx
4	M	79	A	UnRx	35.8	18.6	22.26	6.1	0.65	N-ID	1992	UnRx
5	M	67	B	UnRx	34.0	21.56	15.71	2.74	n/a	n/a	1996	UnRx
6	F	71	B	T	24.0	11.83	28.31	2.74	n/a	n/a	1996	Chl
7	M	80	A	UnRx	17.0	15.71	12.62	3.25	0.51	M	1999	UnRx
8	M	68	B	T	20.0	26.35	19.42	1.01	n/a	n/a	n/a	Chl;Flu;GR;CFM
9	M	74	B	UnRx	70.0	17.4	10.06	2.04	0.57	M	1997	UnRx
10	M	57	A	UnRx	100.0	37.36	35.06	2.39	0.52	M	1993	UnRx
11	M	80	n/a	n/a	80.0	19.41	5.25	2.59	n/a	n/a	2001	n/a
12	F	81	A	UnRx	23.6	20.85	26.35	1.62	n/a	n/a	2001	UnRx
13	M	63	C	T	128.6	9.42	-1.8	1.3	n/a	n/a	n/a	Chl
14	M	71	B	UnRx	39.0	34.48	25.01	5.78	0.56	M	1990	UnRx
15	M	55	B	T	70.0	-3.66	9.42	1.5	0.54	M	1994	Chl
16	M	68	A	UnRx	32.8	14.95	18.69	2.87	0.64	M	1992	UnRx
17	M	64	A	UnRx	70.0	18.69	22.26	1.23	n/a	n/a	2001	UnRx
18	M	59	B	UnRx	70.0	12.62	20.14	0.93	0.64	UM	1997	UnRx
19	F	68	A	UnRx	30.0	0	0	0.65	0.53	M	1999	UnRx
20	M	69	A	UnRx	7.0	0	4.4	1.69	n/a	UM	2001	UnRx
21	M	58	A	UnRx	37.0	-10.4	6.94	0.82	n/a	n/a	2001	UnRx
22	M	56	A	UnRx	20.0	4.4	6.1	0.88	n/a	n/a	2001	UnRx
23	M	60	B	T	96.0	-4.6	-10.43	0.62	0.54	M	1993	Chl x3; PR
24	M	64	A	UnRx	25.0	4	7	0.92	0.65	N-ID	2001	UnRx
25	M	86	A	UnRx	31.0	10.23	12.62	1.73	n/a	n/a	1999	UnRx
26	F	77	C	T	65.4	0	-1.82	0.77	n/a	n/a	1999	n/a
27	M	75	A	UnRx	35.0	16.46	14.95	1.89	0.91	UM	1997	UnRx
28	M	71	A	T	104.3	35.06	24.33	2.08	0.38	N-ID	1991	Chl; PR
29	M	81	C	T	48.3	12.62	11.04	2.81	0.73	UM	2000	Chl x4
30	M	64	A	T	22.9	17.95	-3.66	1.86	0.54	n/a	2001	Pred/CFM;Flu;Rit
31	M	71	C	T	34.7	17.95	14.18	0.92	0.60	n/a	1999	Chl; Flu
32	M	69	A	UnRx	44.1	17.21	2.66	0.78	0.60	M	1980	UnRx
33	M	69	C	T	70.0	17.21	0	0.74	n/a	M	1997	Chl; GR
34	M	62	B	UnRx	53.1	10.23	0	0.64	0.57	n/a	1988	UnRx
35	F	71	B	T	36.3	0	0.9	0.97	1.00	M	1998	Chl;Flu;Pred;Ritm
36	F	83	A	UnRx	26.0	-1.81	6.1	0.52	0.52	M	1998	UnRx
37	M	65	A	T	21.1	26.68	28.31	0.92	0.43	UM	1994	Chl x2
38	M	69	B	T	70.4	-4.6	-15.48	0.49	0.54	M	2002	Chl
39	M	82	n/a	UnRx	34.0	6.1	9.42	1.2	n/a	n/a	2002	UnRx
40	M	79	B	T	19.3	3.53	11.04	0.84	0.61	M	1996	Chl
41	M	77	C	T	52.0	2.66	18.69	0.97	n/a	n/a	1993	Chl
42	F	63	C	T	40.6	8.6	-7.46	0.67	0.83	UM	1990	Chl x3
43	M	55	A	UnRx	37.1	-9.41	-17.47	0.47	0.66	M	1995	UnRx
44	M	72	C	T	50.0	11.94	15.07	1.42	1.30	UM	1994	Chl x6
45	F	62	A	T	25.0	6.94	-14.45	0.54	0.58	N-ID	1998	Chl x2
46	M	79	A	UnRx	n/a	-1.8	11.44	0.31	1.01	n/a	2002	UnRx
47	M	72	A	UnRx	31.0	15.32	16.07	2.62	1.46	UM	2002	UnRx
48	M	53	B	T	75.0	10.69	-35.71	1.51	0.68	M	2000	Chl;CFM;Flu
49	F	62	B	T	27.3	6.94	-2.22	0.91	n/a	n/a	1997	CFM
50	F	84	B	T	100.0	8.59	1.43	0.96	1.43	n/a	1983	Chl;CFM;Flu
51	F	92	A	UnRx	19.5	39.59	13.43	2.35	n/a	n/a	1996	UnRx
52	F	84	C	T	47.0	1.78	0	1.26	n/a	n/a	1994	Chl
52					n/a	0	0	1.26				
52					n/a	6.1	1.78	1				
52					102.0	3.83	3.03	1.98				
53	M	72	A	UnRx	36.1	22.97	14.95	2.18	0.40	n/a	1999	UnRx
53					29.3	15.71	5.25	1.6				
54	M	58	C	T	29.8	16.46	8.6	1.41	n/a	n/a	n/a	Chl
54					30.0	0	34.45	3.16				
55	M	54	A	UnRx	33.2	0	0	2.11	0.58	M	1999	UnRx
55				UnRx	n/a	0.9	4.4	1.31	0.58	M	1999	UnRx
56	M	74	B	T	135.6	30.22	32.07	3.13	n/a	n/a	1998	Chl;Flu;CFM
56					79.0	34.47	27.66	3.31				
57	F	77	B	T	23.4	3.53	11.04	1.26	0.95	UM	1992	Chl
57					n/a	11.45	6.94	1.12				
58	M	75	A	UnRx	30.0	-3.66	7.78	1.13	0.79	UM	1998	UnRx
58					115.0	16.53	-24.06	1.6				
59	M	72	A	T	60.0	11.83	14.18	1.63	n/a	n/a	1999	
59					9.0	5.24	-15.48	1.03				
60	M		A	UnRx	20.0	15.71	25.68	1.55	1.16	UM	1999	UnRx
60		62			42.0	15.71	9.42	2.17				
60					n/a	11.04	33.88	1.14				
61	M	93	A	UnRx	64.3	11.83	15.71	0.77	0.71	M	2001	UnRx
61					n/a	-3.19	-14.98	1.38				

Key: n/a = data not available; UnRx = Untreated; T = Treated; M = Mutated; UM = Unmutated; N-ID = No clone identified. Coloured entries denote a positive MDR result.

3. MM Patient Data

Patient	Age	Sex	ZHCL	VPM	MRK16	%PC	PP type	PP g/l	P/T	Rx	Resp
1	88	F	20.14	n/a	1.00	n/a	n/a	n/a	P	Melph	GR
2	n/a	F	6.10	n/a	1.07	n/a	n/a	n/a	n/a	n/a	n/a
3	62	F	0.00	0.00	0.97	38	IgA/k	24	P	CFM; Pred	PR
4	51	M	6.10	8.60	1.13	22	IgG/k	16	P	CFM; Pred	NR
5	49	M	14.18	n/a	1.39	80	IgD/l	n/a	T	Thal; Dexa	PR
6	n/a	M	0.00	n/a	0.99	n/a	n/a	n/a	n/a	n/a	n/a
7	58	M	4.40	-1.81	1.02	6	IgG/k	53	P	n/a	Died
8	74	M	9.83	10.63	1.01	n/a	IgG/k	23	P	CPM; Pred	PR
9	74	M	14.95	n/a	0.90	15	IgA/k	2	P	Dexa	Died
10	73	M	14.18	-13.42	1.12	40	IgG/k	28	P	IDEX	PR
11	71	M	6.1	3.53	1.08	4	n/a	n/a	T	CFM; Dexa; Thal; Melp	n/a
12	84	F	12.22	8.6	1.18	6	IgA/k	8	T	Radio	NR
13	74	M	18.09	26.34	1.23	17	IgG/k	46	P	Melph; Pred	PR
14	67	M	-3.23	-14.46	0.92	6	k	n/a	T	Radio; CIDEX	PR
15	73	F	10.03	10.77	1.53	14	IgA/k	15	T	CIDEX	NR
16	65	M	6.94	-2.74	1.18	80	IgG/k	73	P	CFM; Thal; CIDEX	PR
16			-1.81	-10.4	1.09	46	IgG/k	73	T		

Key: n/a = data not available; P = presentation; T = Treated; GR = Good Response; NR = No Response; PR = Partial Response; PP = paraprotein; PC = plasma cells; Melph = Melphalan; CFM = cyclophosphamide; Pred = Prednisolone; Thal = Thalidomide; Dexa = Dexamethasone; CIDEX = lomustine, idarubicin and dexamethasone. The coloured entries denote a positive MDR result.

Publications Related To This Thesis

Papers

Gerrard G, Payne E, Baker RJ, Jones DT, Potter M, Prentice HG, Ethell M, McCullough H, Burgess M, Mehta AB, Ganeshaguru K. Clinical Effects and P-glycoprotein Inhibition in Patients with Acute Myeloid Leukemia Treated with Zosuquidar Trihydrochloride, Daunorubicin and Cytarabine. Haematologica. 2004; 89(7):782-90

Abstracts

G Gerrard, K Ganeshaguru, R Baker, M Potter, HG Prentice, M Burgess, L Herbert, H MCCullough, AB Mehta. (2001). A Phase 1 Study of a P-gp Inhibitor Zosuquidar (LY335979), given by short intravenous infusion in Combination with Daunorubicin and Cytarabine in AML/MDS Patients: Preliminary Results of Pharmacodynamic Analysis. *Blood*. 98(suppl.1).

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G Gerrard, K Ganeshaguru, R Baker, M Potter, HG Prentice, M Burgess, L Herbert, H MCCullough, AB Mehta. (2002). Inhibition of P-gp Activity in AML Patients Treated with Zosuquidar Trihydrochloride (LY335979): *In vitro* Data from a Phase 1 Trial. *Blood*. 100(11): 67a.

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G Gerrard, TD Butters, AB Mehta, M Burgess, AV Hoffbrand, D Hughes, K Ganeshaguru. Glucosylceramide Synthase Inhibitors Sensitise B-CLL Cells to Cytotoxic Agents without Reversing P-gp Functional Activity. (2004). *Blood* 104(11): 334a